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Ion channels in the human myometrium.

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Ion Channels in the Human Myometrium

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ABSTRACT

The uterine smooth muscle, or the myometrium, provides the powerful rhythmic contractions required for parturition. Myometrial contractility is associated with changes in membrane potential which in turn are brought about by the movement of ions through membrane-spanning channels. In this study, voltage-gated Ca^{2+} and K^{+} ion channel currents were measured and characterised in freshly dispersed human myometrial smooth muscle cells (HMSMCs), obtained from non-labouring pregnant women at caesarean section, using the whole cell patch clamp technique.

The Ca^{2+} channel current (I_{Ca}) in HMSMCs had a fast, low threshold (T-type) component and a slow high threshold (L-type) component. The cyclooxygenase-II inhibitor and tocolytic agent, nimesulide, inhibited both L- and T-components of I_{Ca} in HMSMCs, as well as spontaneous myometrial contractions *in vitro*. Nimesulide inhibited L-type channels predominantly in resting and inactivated states, in a prostaglandin-independent manner and with greater potency at lower pH. Block of myometrial I_{Ca} by nimesulide may contribute to its myometrial relaxant ability *in vitro* and to its effectiveness as a tocolytic agent *in vivo*.

Three voltage-gated K^{+} channel currents, I_{K1} , I_{K2} and $I_{\text{K,A}}$, were identified in HMSMCs. I_{K1} was a delayed rectifier with a low threshold of activation and inactivation, slow activation kinetics, very slow inactivation kinetics, and was blocked by clofilium and TEA but not 4-AP. I_{K2} was a delayed rectifier with a high threshold of activation and inactivation, faster activation and inactivation kinetics than I_{K1} and was blocked by clofilium and TEA and, in contrast to I_{K1} , also by 4-AP. $I_{\text{K,A}}$ was an A-like current with a low threshold of activation and inactivation, very fast activation and inactivation kinetics, and was inhibited by 4-AP and Cd^{2+} but not TEA. The characteristics of these currents suggest possible functions in the control of slow wave depolarisations and action potential generation and hence myometrial contraction.

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LIST OF ABBREVIATIONS

1). channels, currents and electrophysiological terms

BK_{Ca}	large conductance Ca^{2+} -activated K^+ channel
BK	large conductance Ca^{2+} -insensitive K^+ channel
C_m	membrane capacitance
E_r	potential at which channel current reverses direction
I_m	membrane current
I_{Ba}	Ba^{2+} current
I_{Ca}	Ca^{2+} current
$I_{Cl,Ca}$	Ca^{2+} -activated Cl^- current
I_K	delayed rectifier K^+ current
I_{K1}	low threshold delayed rectifier K^+ current in myometrium
I_{K2}	high threshold delayed rectifier K^+ current in myometrium
$I_{K,A}$	A-like K^+ current
$I_{K,ATP}$	ATP-sensitive K^+ current
$I_{K(Ca)}$	Ca^{2+} -activated K^+ current
I_{Na}	Na^+ current
$I-V$	current-voltage relationship
k	Boltzmann function slope factor
K_{ATP}	ATP-sensitive K^+ channel
K_{IR}	inward rectifier K^+ channel
K_V	voltage-gated K^+ channel
K_I	affinity of drug for inactivated state of channel
K_R	affinity of drug for resting state of channel
R_m	membrane or input resistance
R_s	series or pipette resistance
τ	time constant of current kinetics
$V_{0.5}$	half-inactivation potential

VDCC	voltage-dependent Ca^{2+} channel
V_H	holding potential
V_m	membrane potential

2). drugs, chemicals, enzymes etc.

ANOVA	analysis of variance
4-AP	4-aminopyridine
BAPTA	1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid
$[\text{Ca}^{2+}]_i$	intracellular Ca^{2+} concentration
ChTX	charybdotoxin
COX	cyclooxygenase enzyme
COX-1	type I (constitutive) cyclooxygenase
COX-2	type II (inducible) cyclooxygenase
CPA	cyclopiazonic acid
DMSO	dimethylsulphoxide
EC_{50}	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
HMSMC	human myometrial smooth muscle cell
h-MTC cells	human medullary thyroid carcinoma cells
IbTX	iberiotoxin
IC_{50}	half-maximal inhibitory concentration
INDO	indomethacin
IP_3	inositol 1,4,5, trisphosphate
NIM	nimesulide
NS	not significant

NSAID	non-steroidal anti-inflammatory drug
OT	oxytocin
PBS	phosphate buffered saline
PGE ₂	prostaglandin E ₂
PGF _{2α}	prostaglandin F ₂ -alpha
PGI ₂	prostaglandin I ₂ or prostacyclin
pH _i	intracellular pH
pH _o	extracellular pH
pK _a	dissociation constant
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PSS	physiological saline solution
S.E.M.	standard error of the mean
SR	sarcoplasmic reticulum
TEA	tetraethylammonium
TTX	tetrodotoxin

Chapter 1

Introduction

1.1. GENERAL INTRODUCTION

1.1.1. THE MYOMETRIUM

The uterus is a muscular organ that remains quiescent during gestation, grows as the fetus grows, and at parturition (or labour), produces powerful rhythmic contractions to expel the fetus. In pregnancy, the uterus comprises three parts: the fundus or uterine body, the isthmus or lower segment, and the cervix. The myometrium, the smooth muscle of the uterus, consists of more than one population of smooth muscle fibres, the organisation of which differs between species. In pregnant humans there is an external layer of which there are two sub-layers, one (internal) with circular fibres and the other (external) with longitudinal fibres; an intermediate layer where fibres are diagonally interlaced; and an internal layer of circular fibres. At term, the external longitudinal layer is localised to the fundus, whereas the inner circular layer is predominant in the isthmic region (Devedeux *et al.*, 1993).

The myometrium is an excitable tissue that is capable of spontaneous or myogenic activity. During most of pregnancy however, this activity is suppressed or reduced until, at term, during parturition and post partum, motility is augmented. This inherent spontaneous activity is regulated and modified by endogenous and exogenous factors that include the steroid hormones estrogen and progesterone and the uterotonins oxytocin and prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$).

1.1.2. THE INITIATION OF LABOUR

Although much is now understood about the mechanisms underlying uterine contractility, and it is known that labour is associated with an increased production of prostaglandins* within the uterus (Skinner & Challis, 1985; Romero *et al.*, 1988), the processes through which labour is triggered in man are still not fully understood. This contrasts with sheep

*and increased oxytocin receptors and gap junctions.

where a specific hormonal trigger has been identified. This is known as the fetal hypothalamo-pituitary-adrenal axis, whereby an increased cortisol secretion by the fetal adrenal gland stimulates a switch from progesterone to estrogen production in the placenta, which then stimulates $\text{PGF}_{2\alpha}$ synthesis in the uterus, thus triggering labour (Schellenberg & Liggins, 1994). The substrate for cortisol action in sheep (placental 17-alpha hydroxylase) is absent in man and no other connecting hormonal pathway between elevated fetal cortisol and prostaglandin synthesis in the uterus has been found.

Three principle prostaglandins are produced in the uterus during pregnancy: PGE_2 in the amnion, PGE_2 and $\text{PGF}_{2\alpha}$ in the decidua and PGI_2 (prostacyclin) in the myometrium (Norwitz *et al.*, 1992; Lobaccaro-Henri *et al.*, 1996; Kobayashi *et al.*, 1998; Arntzen *et al.*, 1998). PGE_2 and $\text{PGF}_{2\alpha}$ are stimulatory, via phospholipase C (PLC) and PGI_2 is inhibitory, via adenylate cyclase (Senior *et al.*, 1993; Asboth *et al.*, 1996). Oxytocin (OT) may be involved in the initiation of labour. OT is the most potent natural agent known to elicit uterine contractions. Its effects are two-fold: as well as a direct contractile action via PLC and receptor-gated ion channels, it stimulates prostaglandin production. Although plasma levels of OT do not rise sharply in the early stages of labour, there is a marked increase in OT receptor density in the decidua and myometrium towards term and during labour (Fuchs *et al.*, 1984; Kimura *et al.*, 1992).

Recent studies have suggested a role for G proteins in the initiation of labour. G proteins are the transduction link between agonists such as OT, prostaglandins and noradrenaline and effectors such as PLC, adenylate cyclase and ion channels (see Fig 1.5.). In rats, the expression of G protein subunits during pregnancy is such that agonists stimulating adenylate cyclase are favoured over those that stimulate PLC, thus promoting quiescence (Lopez-Bernal *et al.*, 1995). In humans, $\text{G}\alpha_s$, $\text{G}\alpha_i$, $\text{G}\alpha_q$ and $\text{G}\alpha_z$ are all expressed in myometrium. Expression of $\text{G}\alpha_i$, $\text{G}\alpha_q$ and $\text{G}\alpha_z$ do not change in labour or pregnancy,

whereas $G\alpha_s$ is increased during pregnancy (Europe-Finner *et al.*, 1993), and then reduced again in labour (Europe-Finner *et al.*, 1994).*

1.1.3. MYOMETRIAL CONTRACTILITY

The excitability and rhythmic spontaneous contractions of the uterus are intimately associated with rhythmic bursts of electrical activity initiated by anatomically indistinct pacemaker cells within the uterus (Lodge & Sproat, 1981; Harding *et al.*, 1982; Parkington *et al.*, 1988; Buhimschi *et al.*, 1997; Parkington *et al.*, 1999). This relationship changes during pregnancy and especially during labour and parturition when, in conjunction with increased expression of gap junctions, which allow electrical coupling between adjacent cells (Sims *et al.*, 1982; Miller *et al.*, 1989; Sakai *et al.*, 1992), co-ordinated contraction of the entire uterus is enhanced (Buhimschi *et al.*, 1997).

As was first described for the squid giant axon (Hodgkin & Huxley, 1952) and later for smooth muscle (Bulbring & Tomita, 1970), myometrial electrical activity at the tissue level and membrane potentials at the cellular level result from changes in the permeability of the plasma membrane to various ions, including sodium (Na^+), calcium (Ca^{2+}), potassium (K^+), and chloride (Cl^-), via the opening and closing of ion-selective membrane-spanning ion channels. Compared with other excitable tissues, relatively little is known about the types of ion channel currents in the myometrium and their respective functions in uterine contractility.

In this chapter, current knowledge of ion current characteristics in myometrium, their function with respect to action potentials and excitation-contraction coupling, and knowledge concerning their involvement in myometrial quiescence during pregnancy and its ultimate activity during parturition is described.

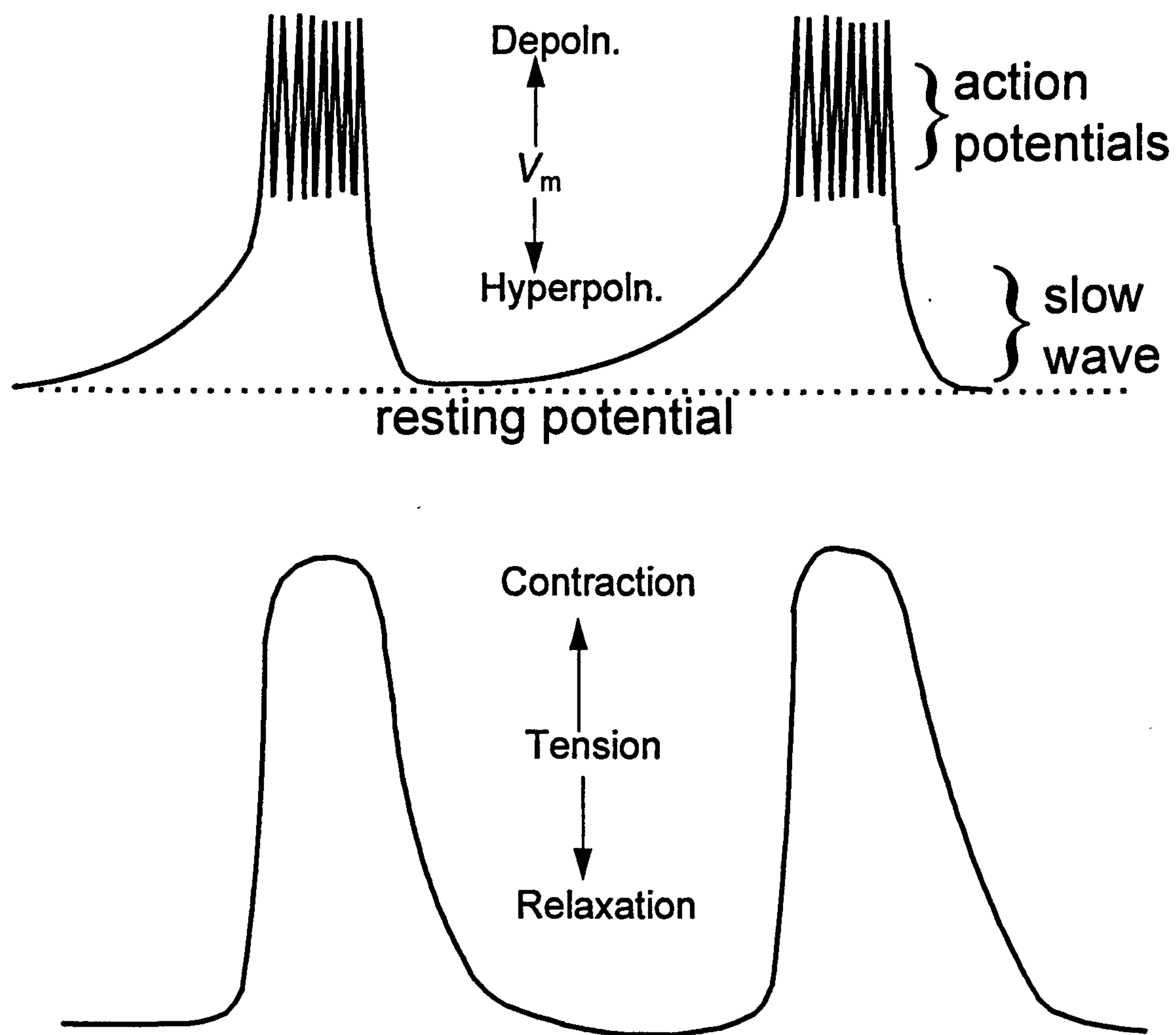
1.2. MEMBRANE POTENTIALS IN THE MYOMETRIUM

The membrane potentials that comprise uterine electrical activity at the muscle bundle or single cell level have two components: the passive component or resting potential and the active component, which itself is composed of slow waves and action potentials (Parkington & Coleman, 1990) (Fig 1.1.).

1.2.1. RESTING MEMBRANE POTENTIALS

The excitability of the cell is influenced by the resting membrane potential. This is the potential difference between the net negative charge inside the cell and net positive charge outside. Depolarisation or hyperpolarisation of the resting membrane potential, brings the cell either closer to or further away from the threshold for the triggering of action potentials, respectively. Resting membrane potentials of spontaneously active smooth muscles, including the myometrium, are generally smaller (ie less negative) than those of non-spontaneous tissues such as most vascular smooth muscle (Burnstock *et al.*, 1963). Some measurements of myometrial resting membrane potentials in term non-labour myometrium are listed in Table 1.1.

FIG 1.1. RELATIONSHIP BETWEEN MEMBRANE POTENTIAL AND CONTRACTION



Drawing of slow waves, action potentials and associated contractions in pregnant human myometrium, based on data in Kowarabayashi (1986b), Inoue *et al.*, (1990) and Nakao *et al.* (1997).

In three of the studies listed in Table 1.1. resting membrane potential in rats was also measured during early and mid pregnancy (but not in non-pregnant) and was on average more negative (by 5-10 mV) than at term (Anderson *et al.*, 1981; Kuriyama & Suzuki, 1976a; Lodge & Sproat, 1981). This early sustained hyperpolarisation of the myometrium renders the tissue less electrically excitable and therefore contributes to its quiescence, whereas the depolarisation towards the end of pregnancy increases its excitability and probably contributes to its preparation for contractility.

TABLE 1.1.

Resting Membrane Potentials in Pregnant Myometrium (mV)					
Species	mV	Author(s)	Species	mV	Author(s)
Rat	-36 to -46	Anderson <i>et al.</i> (1981)	Mouse	-45	Osa (1974)
Rat	-41	Kao & McCullough (1975)	Sheep	-50	Parkington (1985)
Rat	-54	Kuriyana & Suzuki (1976a)	Human	-49	Pressman <i>et al.</i> (1988)
Rat	-56	Lodge & Sproat (1981)	Human	-51	Nakao <i>et al.</i> (1997)
Rat	-55	Mollard <i>et al.</i> (1986)	Human	-65	Kumar & Barnes (1961)
Rat	-50 to -60	Amadee <i>et al.</i> (1987)	Human	-50	Inoue <i>et al.</i> (1990)
Rat	-50	Miyoshi <i>et al.</i> (1991)	Human	-47	Anwer <i>et al.</i> (1993)
Rat	-55 to -65	Casteels & Kuriyama (1965)	Human	-45	Nakajima (1971)
Guinea-pig	-50	Coleman & Parkington (1987)	Human	-59	Parkington <i>et al.</i> (1999)

1.2.2. SLOW WAVES

Slow waves, also known as pacemaker pre-potentials or pacesetter potentials (Marshall, 1962; Reiner & Marshall, 1975; Kuriyama & Suzuki, 1976a; Lodge & Sproat, 1981) are characteristic regular oscillations of the membrane potential and occur either spontaneously, or in response to agonist binding or current injection that depolarises the resting membrane potential. Slow wave amplitude is small (2-10 mV, depending on initial resting level, Nakao *et al.*, 1997) and frequency varies from once every several seconds to once a minute. Slow wave frequency (determined by the rate of rise of the slow depolarisation) determines the frequency of contractions (Reiner & Marshall, 1975; Kuriyama & Suzuki, 1976a). In pregnant human myometrium, slow wave depolarisations alone are not sufficient to trigger contractions. Contractions occur when the slow depolarisation reaches the threshold for regenerative action potential generation (Kawarabayashi *et al.*, 1986a).

1.2.3. ACTION POTENTIALS

The amplitude of spontaneous or stimulated regenerative action potentials in pregnant rat and sheep myometrium is commonly large, around +50 mV with a positive overshoot of around 10 mV (Kao & McCullough, 1975; Mollard *et al.*, 1986; Parkington, 1985). In pregnant human myometrium, action potential amplitudes are variable, having been recorded to be as small as only 4 mV (Kawarabayashi *et al.*, 1986a), between 10 and 20 mV (Young *et al.*, 1991; Nakao *et al.*, 1997) or of similar magnitude as those in the rat (Inoue *et al.*, 1990; Parkington *et al.*, 1999). The commonest form of action potential in smooth muscle is the simple spike which occurs in rapid bursts superimposed on each slow wave. The frequency of spikes in these bursts is in the order of 10 Hz (Kuriyama & Suzuki, 1976a). In general, the frequency and amplitude of spike potentials determine the amplitude and duration of contractions.

Sometimes bursts of short spikes are replaced by complex action potentials, which comprise an initial spike or series of spikes, followed by a sustained plateau depolarisation (Vassort, 1975; Parkington, 1984). In rats and guinea-pigs, the occurrence of simple spike vs. complex action potentials varies during gestation and between muscle layers (Parkington & Coleman, 1988; Anderson *et al.*, 1981; Parkington, 1983; Bengtsson *et al.*, 1984; Kawarabayashi & Marshall, 1981; Wilde & Marshall, 1988). The association of complex action potentials with contractions is also variable (Parkington & Coleman, 1988). In term pregnant human myometrium, simple spike, plateau or complex plateau with spike types of action potentials may all be observed, either in bundles of smooth muscle cells (Kawarabayashi *et al.*, 1986a; Nakao *et al.*, 1997; Parkington *et al.*, 1999), or in single freshly dispersed cells (Inoue *et al.*, 1990).

1.2.4. IONIC NATURE OF MEMBRANE POTENTIALS

Resting Membrane Potential

The resting membrane potential is the result of opposing inward (Na^+ , Ca^{2+}) and outward (K^+ , Cl^-) ionic fluxes. The importance of Na^+ to the resting membrane potential in rat and human myometrium is demonstrated by the hyperpolarisation resulting from removing external sodium (Kao & McCullough, 1975; Mollard *et al.*, 1986; Inoue *et al.*, 1990; Parkington *et al.*, 1999). By contrast, it has been shown that inhibition of K^+ efflux by loading pregnant rat myometrial strips with Cs^+ or by applying the K^+ efflux blockers 4-aminopyridine (4-AP) and iberiotoxin, causes a steady depolarisation of the resting membrane potential sufficient to trigger action potential generation (Jmari *et al.*, 1986a; Wilde & Marshall, 1988; Anwer *et al.*, 1993). Also, in pregnant human myometrium strips, 2 mM tetraethylammonium (TEA) caused a 5-10 mV depolarisation of the resting membrane potential (Parkington *et al.*, 1999). The resting membrane potential steadily hyperpolarises as gestation progresses and then depolarises in the days preceding labour, and in the rat it has been suggested that these changes are caused by increases in K^+ permeability and increases in Na^+ permeability, respectively (Casteels & Kuriyama, 1965).

Slow Waves

The ionic nature of this slow depolarisation has been partially characterised. In rats, it is associated with an increased membrane resistance which is attributed to a decrease in K^+ permeability (Kuriyama & Suzuki, 1976a). The slow depolarisation also disappears when external Na^+ is removed suggesting that Na^+ influx is involved (Reiner & Marshall, 1975). In isolated pregnant human myometrial cells, the passive electrotonic response to hyperpolarisation was enhanced in Ca^{2+} -free and Na^+ deficient solution suggesting that membrane excitability is influenced by both Ca^{2+} and Na^+ (Inoue *et al.*, 1990). Both inward and outward movement of ions is therefore involved in slow wave depolarisations and the size and slope of the slow wave probably results from the shifting balance between slow inward currents and outward rectifying currents (Wilde & Marshall, 1988). In murine colonic smooth muscle (a rhythmic tissue), 4-aminopyridine, a blocker of voltage-gated K^+ channels (see section 1.4.) inhibited slow wave repolarisation, producing a continuous depolarisation (Koh *et al.*, 1999).

Spike-Type Action Potentials

Electrically-induced contraction of the rat myometrium requires Ca^{2+} influx (Mironneau, 1973). Furthermore, the overshoot amplitude of spontaneous or electrically stimulated action potential spikes in single cells of rat and human myometrium and strips of sheep myometrium is dependent on external Ca^{2+} concentration, but not that of external Na^+ , and is reduced by Ca^{2+} influx blockers (divalent cations and dihydropyridines), but not blockers of Na^+ influx (tetrodotoxin) even after prolonged hyperpolarising current injection to reactivate inactivated Na^+ channels (Kuriyama & Suzuki, 1976a; Kao & McCullough, 1975; Bengtsson *et al.*, 1984; Mollard *et al.*, 1986; Amedee *et al.*, 1986b; Parkington, 1985; Inoue *et al.*, 1990; Parkington *et al.*, 1999). The regenerative spike potential therefore involves mostly Ca^{2+} entry.

Repolarisation of the spike potential involves a reduction of the Ca^{2+} influx via inactivation and closing of Ca^{2+} channels and an increased K^{+} efflux. Ca^{2+} channel inactivation and hence rate of spike repolarisation is dependent on both membrane potential and intracellular calcium concentration. The intracellular Ca^{2+} dependence is illustrated by replacing external Ca^{2+} with Ba^{2+} or Sr^{2+} which lengthens the duration of action potentials in pregnant rat myometrium (Jmari *et al.*, 1986; Amedee *et al.*, 1987). These mechanisms are detailed later in this chapter.

Evidence for the involvement of K^{+} efflux was provided by experiments in strips and single cells of pregnant rat myometrium. Loading the cells with Cs^{+} increases action potential amplitude when the depolarisation of the resting membrane potential was compensated for by current injection (Jmari *et al.*, 1986). Furthermore, when cells are not perfused with Cs^{+} to block the K^{+} currents, K^{+} efflux blockers TEA and 4-AP increase the amplitude and duration of action potentials (Mollard *et al.*, 1986). A similar increased amplitude in prostaglandin-induced plateau type action potentials was caused by the K^{+} efflux blockers charybdotoxin and 4-AP in strips of pregnant human myometrium (Parkington *et al.*, 1999).

The K^{+} efflux itself may also be dependent on Ca^{2+} influx. This is implied by the reduction in outward rectification under current clamp conditions by the addition of TEA and removal of external Ca^{2+} in strips of human myometrium (Pressman *et al.*, 1988), and the fact that one of the K^{+} efflux channels is activated by intracellular Ca^{2+} (Coleman & Parkington, 1987).

Complex Action Potentials

In complex action potentials of pregnant rat myometrium, the ionic nature of the spike component also involves Ca^{2+} , since it is reduced by removing external Ca^{2+} and by the Ca^{2+} channel blocker D-600 (Wilde & Marshall, 1988). The plateau component, involves a

considerable conductance (Wilde & Marshall, 1988) and in the rat is affected by altering external Cl^- concentration, but not those of Ca^{2+} or Na^+ (Parkington, 1984), but in humans is inhibited by nifedipine and verapamil, blockers of Ca^{2+} entry through voltage-gated channels (Parkington *et al.*, 1999). The duration of the plateau potential in rat myometrium is dependent on external Ca^{2+} concentration, suggesting an inward Ca^{2+} -activated current, possibly involving Cl^- efflux through a large conductance channel, is clamping the plateau potential (Parkington, 1984; Parkington & Coleman, 1988; Arnaudeau *et al.*, 1994). Sustaining the depolarisation at this level maintains a slow Ca^{2+} influx and prolongation of the contraction. In the rat, the amplitude of the plateau phase was also increased by the inhibitor of K^+ efflux, TEA, which also reduces the outward rectification and membrane conductance during the plateau, suggesting the involvement of K^+ efflux-hyperpolarisation opposing the Cl^- efflux depolarisation (Wilde & Marshall, 1988). Eventual repolarisation of the plateau probably involves a decline in the Cl^- efflux and slow Ca^{2+} influx and the predominance of a slow K^+ efflux (Vassort, 1975). In pregnant human myometrial strips, the duration of the $\text{PGF}_{2\alpha}$ or PGE_2 induced plateau potentials was increased by the K^+ channel blockers, Ba^{2+} , 4-AP and charybdotoxin (Parkington *et al.*, 1999).

1.2.5. EFFECTS OF STEROID HORMONES ON MEMBRANE POTENTIALS

The resting membrane potential and hence excitability of the cell, is influenced by steroid hormones. In the spayed rat, pre-treatment (> 24 hrs) with estrogen or progesterone individually hyperpolarises the myometrial resting membrane potential to a level similar to that of the mid-pregnant rat, whereas combined application of the two hormones produces a depolarisation similar to that observed at the end of pregnancy when estrogen levels are high (Kuriyama & Suzuki, 1976a).

Action potentials are also influenced by prior treatment with steroid hormones. In estrogen treated ovariectomised rats complex action potentials are induced (Kuriyama & Suzuki 1976b; Parkington, 1984). Estrogen also increases the action potential amplitude, the length of the burst and the frequency of spikes in a burst (Marshall, 1959). The effects of progesterone on action potentials are dependent on the presence of estrogen, since in the term pregnant rat it induces action potentials similar to those of the mid-pregnant rat (Kuriyama & Suzuki, 1976a). Progesterone also reduces the amplitude and frequency of action potential spikes (Marshall, 1959; Kuriyama & Suzuki, 1976a). How these changes may involve ion channels are discussed in later sections.

1.2.6. EFFECTS OF UTEROTONINS ON MEMBRANE POTENTIALS

The physiologically and clinically important uterotonins, oxytocin (OT) and the prostaglandins PGE₂ and PGF_{2α} have complex and variable effects on membrane potential which are dependent on the concentration or on the gestational and hormonal status. In the rat, responses to OT include increased frequency and number of action potential spikes in a burst, decreased interval between bursts of action potentials and induction of plateau phase action potentials which replace the bursts of spike potentials (Kuriyama & Suzuki, 1976b). In term pregnant human myometrium by comparison, oxytocin at low concentrations evoked plateau phase action potentials and increased the amplitude, frequency and duration of spontaneous plateau potentials (Nakao *et al.*, 1997; Kawarabayashi *et al.*, 1990), whereas at higher concentrations it depolarised the resting membrane potential and reduced membrane resistance (Nakao *et al.*, 1997). In single myometrial cells from pregnant rat in primary culture OT induces depolarisations that are powerful enough to trigger regenerative action potentials (Arnaudeau *et al.*, 1994).

In pregnant mouse myometrium, low concentrations of PGE₂ increased the frequency and number of spikes in a burst of action potentials without affecting the resting or peak

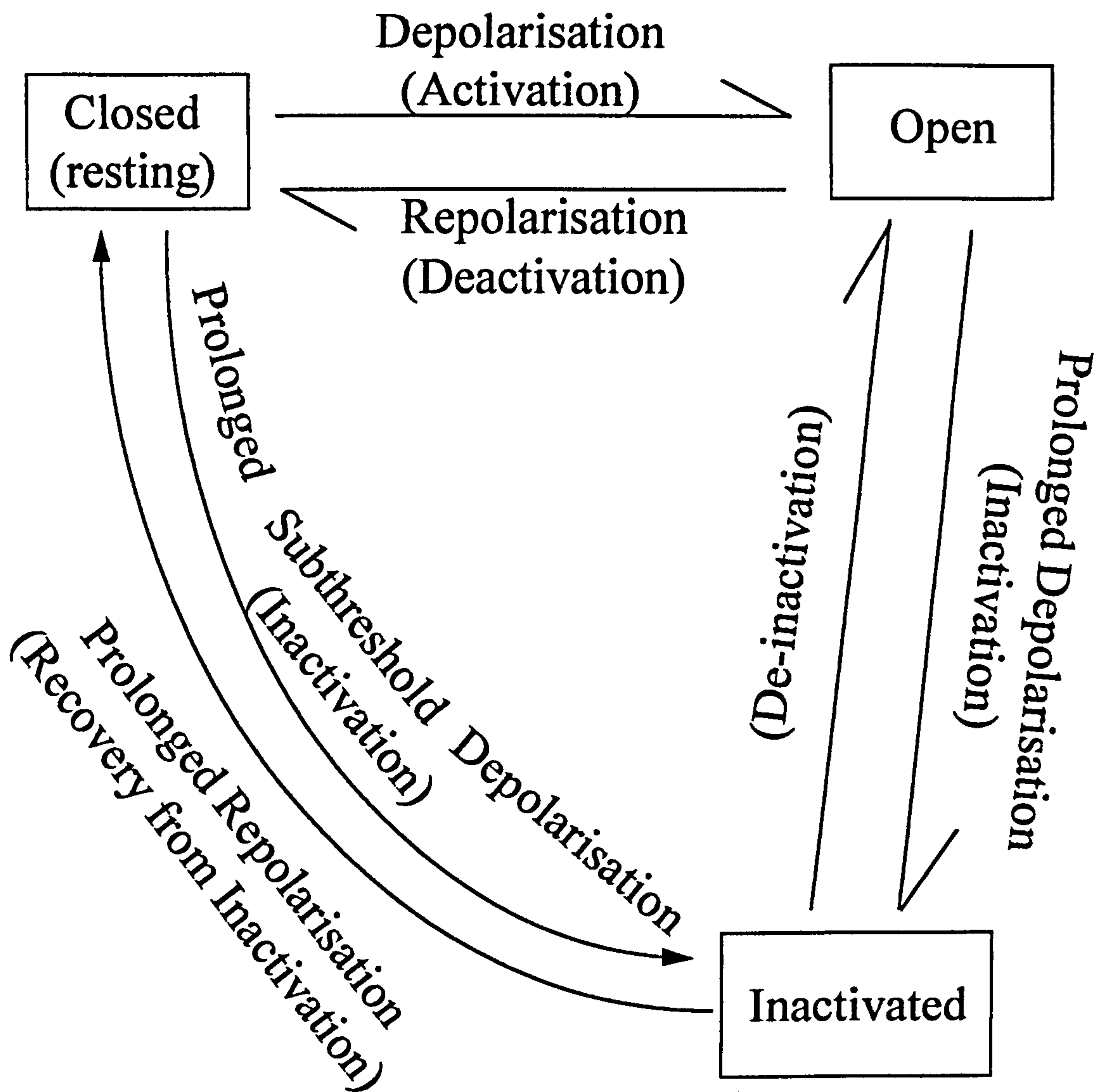
membrane potential, whereas at higher concentration it depolarises the membrane and produces continuous spike generation (Suzuki & Kuriyama, 1975). In pregnant human myometrium, low concentrations of PGE₂ and PGF_{2α} (10-100 nM) evoked depolarisations without triggering action potentials, but at high concentrations (10 μM) they both induced-plateau type action potentials or enhanced the amplitude of spontaneous action potentials and induced large after-hyperpolarisations (Parkington *et al.*, 1999).

1.3. CALCIUM, SODIUM AND CHLORIDE CURRENTS

1.3.1. VOLTAGE-GATED CALCIUM CHANNELS

Ca²⁺ channels exist in most if not all types of excitable cell and are necessary for excitation-contraction coupling in cardiac, skeletal and smooth muscle cells, as well as neurotransmitter and endocrine secretion in nerves and neuroendocrine cells. Voltage-dependent Ca²⁺ (as well as Na⁺ and K⁺) channels exist in three principle states, the closed or resting state, the open state and the inactivated state (Hodgkin & Huxley, 1952) (Fig 1.2.). The characteristics of channel opening, closing and inactivation differ between channel subtypes and determine their current kinetics and voltage-dependencies (see below).

FIG 1.2. THREE PRINCIPLE STATES OF VOLTAGE-GATED ION CHANNELS



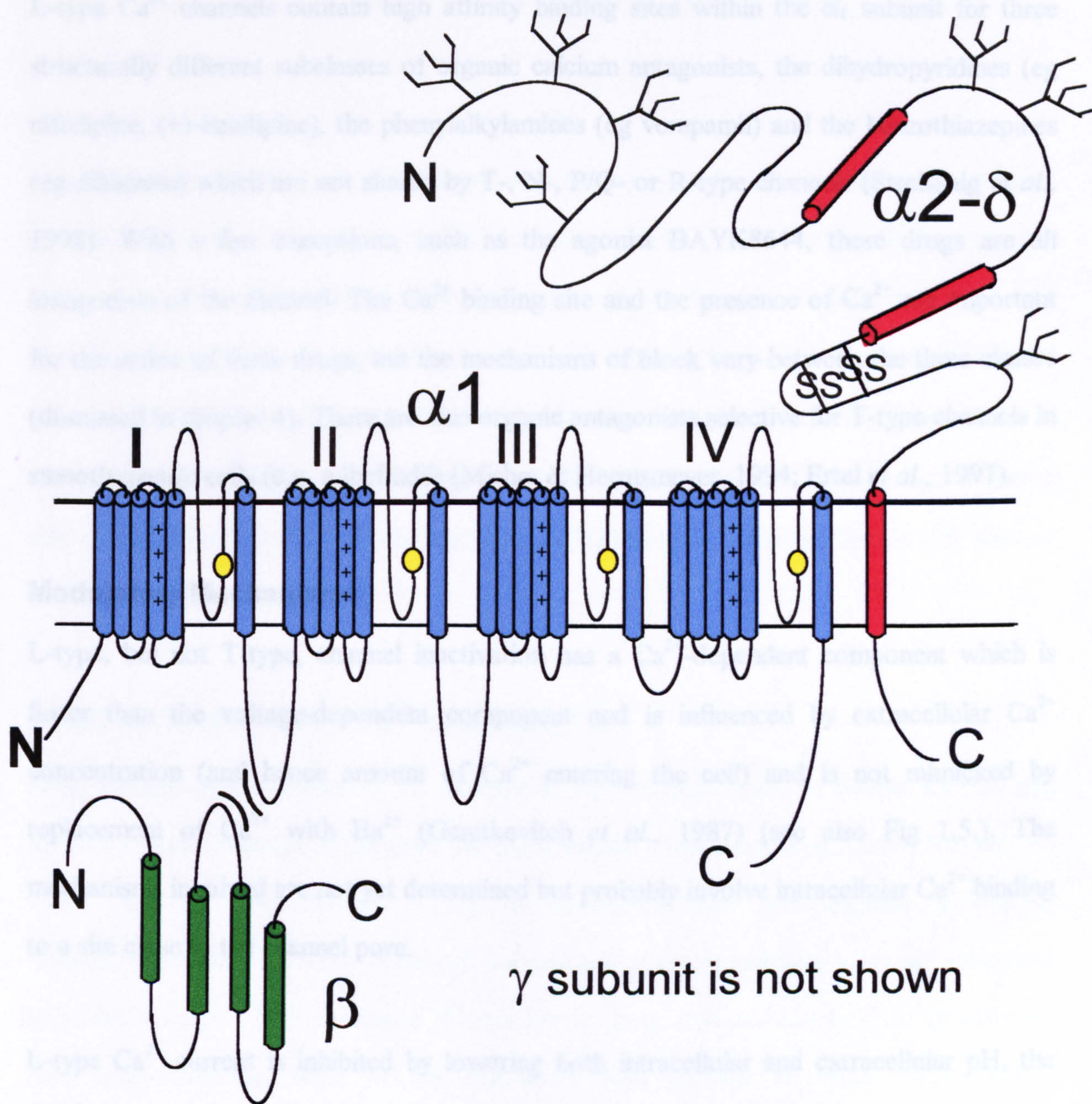
Channels open in response to membrane depolarisation (moving from resting to open states) and upon repolarisation, close, returning to the resting state (also known as deactivation). Prolonged depolarisation however causes channel closing by inactivation, which requires a further period of prolonged repolarisation, during which the channel eventually returns to the resting state, before it is capable of opening again. Prolonged subthreshold depolarisation may also result in inactivation, channels moving directly from resting to inactivated states.

Structure and Voltage-Sensitivity








Voltage-dependent Ca^{2+} channels (VDCCs) are heteromeric complexes of membrane-spanning proteins, which show a high level of electrophysiological and pharmacological diversity. There are at least five classes of VDCC, which to date include L-, T-, N-, P/Q-, and R-type channels. L- and T-type are the predominant VDCC in smooth muscle. The channel unit is comprised of at least 4 classes of subunit, termed α_1 , α_2 - δ , β and γ . The most important of these is the α_1 subunit, which in smooth muscle L-type channels is the cloned $\alpha_1\text{C}$ (CaCH2b) protein (Hoffmann *et al.*, 1994), and in neuronal and cardiac T-type channels, the $\alpha_1\text{G}$ and $\alpha_1\text{H}$ proteins, respectively (Perez-Reyes *et al.*, 1998; Cribbs *et al.*, 1998). These comprise four homologous transmembrane units each composed of six transmembrane domains linked by cytoplasmic loops and with cytoplasmic amino (N) and carboxy (C) termini domains (Fig 1.3.). The α_1 subunit contains the pore with a Ca^{2+} -selective filter which binds one Ca^{2+} ion with high affinity (non-permeate state) or two with low affinity (allowing permeation) (Yang *et al.*, 1993) The α_1 subunit also contains a voltage sensor which controls the voltage-dependence of channel opening. This is contained within the fourth membrane-spanning domain of each repeat and contains positive charged residues at each turn of the helix which move in the extracellular direction on depolarisation thus opening the pore (Catterall, 1995). Inactivation mechanisms are not yet determined but may involve structural rearrangement of the sixth membrane-spanning domain of the first repeat (Zhang *et al.*, 1994).

The membrane anchored largely extracellular α_2 - δ subunit, the cytoplasmic β subunit, and the transmembrane γ subunit interact with the α_1 subunit (see Fig 1.3.), influencing voltage-dependence of activation (often causing hyperpolarising shifts in activation) and kinetics of activation and inactivation. There are at least six α_1 , four β , and one α_2 - δ genes, many of which encode multiple splice variants, accounting for the extensive heterogeneity of native VDCC current characteristics (Walker & De Waard, 1998).

FIG 1.3. STRUCTURE OF VOLTAGE-GATED Ca^{2+} CHANNELS



γ subunit is not shown

	helix of β subunit		voltage sensor
	transmembrane helix of $\alpha 1$ subunit	N	amino terminus
	helix of $\alpha 2$ - δ subunit	C	carboxy terminus
	glutamate residues of Ca^{2+} -selective filter	SS	disulphide bridge
	interaction between $\alpha 1$ and β subunits		glycosylation sites

Pharmacology

L-type Ca^{2+} channels contain high affinity binding sites within the α_1 subunit for three structurally different subclasses of organic calcium antagonists, the dihydropyridines (eg nifedipine, (+)-isradipine), the phenylalkylamines (eg verapamil) and the benzothiazepines (eg diltiazem) which are not shared by T-, N-, P/Q- or R-type channels (Streissnig *et al.*, 1998). With a few exceptions, such as the agonist BAYK8644, these drugs are all antagonists of the channel. The Ca^{2+} binding site and the presence of Ca^{2+} are important for the action of these drugs, but the mechanisms of block vary between the three classes (discussed in chapter 4). There are also organic antagonists selective for T-type channels in smooth muscle cells (e.g. mibefradil) (Mishra & Hermsmeyer, 1994; Ertel *et al.*, 1997).

Modulatory Mechanisms

L-type, but not T-type, channel inactivation has a Ca^{2+} -dependent component which is faster than the voltage-dependent component and is influenced by extracellular Ca^{2+} concentration (and hence amount of Ca^{2+} entering the cell) and is not mimicked by replacement of Ca^{2+} with Ba^{2+} (Ganitkevitch *et al.*, 1987) (see also Fig 1.5.). The mechanisms involved are not yet determined but probably involve intracellular Ca^{2+} binding to a site close to the channel pore.

L-type Ca^{2+} current is inhibited by lowering both intracellular and extracellular pH, the mechanisms of which are still unclear, but probably involve masking of surface charge at or around the site of the pore by H^+ , preventing access of Ca^{2+} to the pore (Ohmori & Yoshii, 1977; Shmigel *et al.*, 1995).

Protein kinases influence Ca^{2+} channel opening in smooth muscle by phosphorylating the channel protein. cAMP-dependent PKA and phospholipase C-dependent PKC both stimulate channel activity, whereas the cGMP-dependent PKG may inhibit (Beech & McHugh, 1996). The requirement for ATP of Ca^{2+} channel activity appears to vary

between cell types, being essential for prevention of current run-down (time-dependent decay) in some, including smooth muscle (Beech & McHugh, 1996; Lorenz & Paul, 1997).

There is evidence for both stimulatory and inhibitory pathways involving G proteins. Agonist stimulation (e.g. by histamine) activates smooth muscle L-type Ca^{2+} current via Gq coupling to phospholipase C, diacylglycerol production and hence PKC activation (see Fig 1.5). β -adrenoceptor stimulation by contrast, inhibits L-type Ca^{2+} current in smooth muscle possibly via direct interaction of the channel with Gs α -subunit, independent of cAMP production (Khac *et al.*, 1992; Beech & McHugh, 1996). There is also evidence that neuronal non-L-type Ca^{2+} channels may be inhibited by direct binding of $\text{G}\beta\gamma$ subunit to the channel (Dolphin, 1998).

1.3.2. MYOMETRIAL Ca^{2+} CURRENTS

Early voltage-clamp studies of ion currents in myometrium used the double sucrose gap method in small bundles of pregnant rat myometrium (Anderson *et al.*, 1971; Mironneau, 1973; Kao & McCullough, 1975) or estrogen-treated non-pregnant guinea-pig myometrium (Vasort, 1975). Although inward and outward currents were not examined individually and the high resistance of these preparations rendered much of the quantitative data uninterpretable, some of the qualitative data remains of value. With normal extracellular Ca^{2+} , Na^{+} and K^{+} concentrations, depolarising voltage steps elicited early transient inward currents followed by slower sustained outward currents (Kao & McCullough, 1975; Vasort, 1975). The amplitude of the transient inward current was attenuated by the reduction of both external Ca^{2+} and Na^{+} concentrations (Anderson *et al.*, 1971; Kao & McCullough, 1975), suggesting the presence of either a Ca^{2+} or a Na^{+} current, or both, in these preparations. However, the near complete inhibition by Mn^{2+} and

D600, suggested that Ca^{2+} was the major charge carrier of this current (Mironneau, 1973; Vasort, 1975).

The inactivation mechanisms of the Ca^{2+} current in pregnant rat myometrium were first investigated by Jmari *et al.* (1986) using the sucrose gap technique in small strips of muscle and blocking the outward K^+ currents with Cs^+ . The Ca^{2+} current was inactivated by conditioning pre-pulses to positive potentials up to 0 mV, but this inactivation was less pronounced at more positive potentials as less Ca^{2+} entered the cell during the conditioning pre-pulse. Replacement of external Ca^{2+} with Ba^{2+} resulted in slower decay or inactivation of the current, whereas the inactivation rate was enhanced by raising the external Ca^{2+} concentration but not by raising the external Ba^{2+} concentration. It was concluded that inactivation of the Ca^{2+} current is sensitive to both voltage and intracellular Ca^{2+} concentration but that inactivation of the Ba^{2+} substituted current was purely voltage-dependent. This current was therefore characteristically of the L-type. The ionic selectivity of the channel carrying this inward current was $\text{Ca}^{2+} \gg \text{Ba}^{2+} = \text{Sr}^{2+} > \text{Na}^+$ (Jmari *et al.*, 1987).

L-Type Ca^{2+} Current

The above described inactivation characteristics and ion selectivity of the L-type Ca^{2+} current in pregnant rat myometrium were later confirmed in single cells using the intracellular microelectrode or patch-clamp methods and with either Cs^+ in the patch pipette or with 4-AP in the external solution (Amedee *et al.*, 1987; Honore *et al.*, 1989a). Furthermore, with 1-2 mM Ca^{2+} in the bath solution, the half-inactivation potential was -38 mV, the activation threshold was between -50 and -35 mV, and the peak amplitude was between -10 and 0 mV (Honore *et al.*, 1989a; Ohya & Sperelakis, 1989; Inoue & Sperelakis, 1991). The amplitude of I_{Ca} , threshold of activation and peak amplitude are all shifted positively by increasing the external Ca^{2+} concentration (Yoshino *et al.*, 1997) whereas replacement with Ba^{2+} shifts these parameters slightly in the negative direction

(Ohya & Sperelakis, 1989; Yoshino *et al.*, 1997). The decay of the L-type current is relatively slow (with respect to other transient inward currents such as T-type Ca^{2+} and fast Na^+), being incomplete within 200 ms at potentials close to the threshold (Ohya & Sperelakis, 1989; Inoue & Sperelakis, 1991). The predominance of the L-type current in pregnant rat myometrium is also confirmed pharmacologically by the near complete inhibition with low concentrations of D-600, nifedipine, isradipine or nisoldipine and the divalent cations Cd^{2+} , Mn^{2+} or Co^{2+} , but not by the specific blocker of voltage-gated Na^+ channels, tetrodotoxin (Amedee *et al.*, 1987; Honore *et al.*, 1989a; Miyoshi *et al.*, 1991; Ohya & Sperelakis, 1989; Inoue & Sperelakis, 1991; Yoshino *et al.*, 1997). The I_{Ca} in pregnant rat myometrium is also enhanced by BAY8644 (Kyojuka *et al.*, 1987; Yoshino *et al.*, 1997). Amplitude of L-type Ca^{2+} current in pregnant rat myometrium is dependent on phosphorylation by enzymes such as tyrosine kinase and protein kinase C (Kusaka & Sperelakis, 1995; Shimamura *et al.*, 1994a).

The first description of L-type Ca^{2+} channels in human myometrium was by Batra & Popper (1989) who demonstrated the presence of high affinity dihydropyridine binding sites in pregnant and non-pregnant human myometrium. This was later confirmed electrophysiologically with the whole cell patch clamp method (see below).

T-Type Ca^{2+} Current

In the study by Honore *et al.* (1989), using a more negative holding potential of -70 mV, a second population of Ca^{2+} currents with a more negative range of inactivation was observed in a small proportion of cells (11 %). They suggested this may represent a T-type Ca^{2+} current. Ohya & Sperelakis (1989) by contrast ruled out the existence of T-type Ca^{2+} current in 18 day pregnant rat myometrium because when using a voltage protocol designed to maximally activate the T-current (holding potential of -90 mV and test potential of -20 mV), the current was completely blocked by a low concentration of

nifedipine. In retrospect, the second population of currents described by Honore *et al.* (1989) may have been the fast Na^+ current.

A Ca^{2+} current has also been described in myometrium of adult non-pregnant rats. In these cells, the current did not have two clearly defined components but possessed a relatively negative inactivation threshold of between -44 mV and -55 mV, was only partially sensitive to 1 μM nifedipine, and was more sensitive to block by Ni^{2+} than by Cd^{2+} (Rendt *et al.*, 1992). Whether this means that the T-type Ca^{2+} current predominates in non-pregnant rat and is then down regulated or replaced by L-type current during pregnancy is unclear. If such changes occur, they may involve the influence of steroid hormones.

In pregnant human myometrium, studies using the single cell patch clamp method have identified two distinct types of Ca^{2+} current, in contrast to the pregnant rat. With Ca^{2+} in the bath solution and Cs^+ in the patch pipette, the resultant inward current was blocked by removing external Ca^{2+} and by low concentrations of nicardipine or D-600, characteristic of the L-type Ca^{2+} current (Inoue *et al.*, 1990). When the holding potential was shifted from -60 to -100 mV however, two populations of current ^{were observed}. The first demonstrated a slow decay, threshold at around -50 mV, and peak amplitude at 0 mV. The second exhibited a faster decay, a lower threshold of below -60 mV and peak amplitude between -30 and -20 mV. The relative peak amplitudes of the two currents were similar in these cells. The slow, high threshold current was present at both holding potentials and represents the L-type current. The fast, low threshold current was only present at the -100 mV holding potential, characteristic of the T-type Ca^{2+} current (Inoue *et al.*, 1990). Young *et al.*, (1991 & 1993) also described two populations of inward current that were both inhibited by Co^{2+} , but in contrast to Inoue *et al.* (1990), found that the fast, low threshold current predominated by a factor of 3-4 fold. Inactivation experiments were performed on these two currents, isolating T-type from L-type with the L-type blocker, nifedipine and different test

potentials. The half inactivation was -70 mV for T-type current and -27 mV for L-type current (Young *et al.*, 1993).

Inoue *et al.* (1990) also performed single channel studies isolating the two currents by adjusting the voltage protocol. From a holding potential of -100 mV, voltage steps up to -20 revealed a 12.2 pS conductance whereas from a holding potential of -60 mV, steps between -30 and +10 revealed a conductance of 28.8 pS. The larger conductance is that of L-type channels. The smaller conductance was still present in 100 mM Ba²⁺ and in the absence of external Na⁺, ruling out the possibility of a fast Na⁺ current, and therefore probably represented the T-type current.

Gestational and Hormonal Effects on Ca²⁺ Currents

As part of the mechanism preparing the uterus for labour, myometrial ion channel expression may be altered. Inoue & Sperelakis (1991) found that the amplitudes of Ca²⁺ current in pregnant rat myometrium increased slightly from day 5 and peaked at day 9. Yoshino *et al.* (1997) however, found that it increased during the first trimester of pregnancy, decrease gradually in the second (days 7-14) and third trimester (days 15-21) up to day 18 and then declined dramatically post-partum. The decline in the second and third trimesters was attributed to a 3-4 fold increase in cell size at this time without a similar increase in current amplitude (Yoshino *et al.*, 1997). Despite the above described fall in current density toward term, mRNA expression of L-Type Ca²⁺ channel α_1 -subunits in pregnant rat myometrium increases gradually during pregnancy to a 7-fold level on day 22 compared to day 1 and then decrease during labour (Mershon *et al.*, 1994; Tezuka *et al.*, 1995). In freshly isolated porcine myometrial cells the amplitude of Ca²⁺ current (using Ba²⁺ as charge carrier) was increased in late pregnancy by 2-3 fold compared with non-pregnant (ZhuGe & Hsue, 1994).

In non-pregnant adult rat, the Ca^{2+} current is larger in diestrus than in estrus and is increased by prior progesterone injection (Rendt *et al.*, 1992). In contrast, progesterone injection during pregnancy inhibits term delivery and prevents the normal increase in Ca^{2+} channel α_1 -subunit expression at this time, whereas antiprogesterone treatment significantly increases expression (Tezuka *et al.*, 1995).

Acutely, 17β -estradiol causes a leftward shift in the inactivation curve of the Ca^{2+} current in rat myometrial cells thus reducing cell excitability (Yamamoto, 1995). This differs from the long term effect of raised circulating estradiol levels at the end of pregnancy, where expression of the channel is increased (Mershon *et al.*, 1994; Tezuka *et al.*, 1995). Furthermore, chronic *in vivo* treatment of non-pregnant ovariectomised rats results in an increase of both ^{45}Ca influx in contracting strips of myometrium and density of nitrendipine binding sites in membrane fractions (Batra, 1987). OT also influences Ca^{2+} current in isolated cells of rat and porcine myometrium, although in some studies it caused an inhibition (Inoue *et al.*, 1992) whereas in others it stimulated (Zhuge & Hsu, 1994; Zhuge *et al.*, 1995).

1.3.3. VOLTAGE-GATED SODIUM CHANNELS

Voltage-gated Na^+ channels are essential for rapid transmission of electrical impulses in neurons as well as excitation-contraction coupling in cardiac and skeletal muscles. Na^+ channels are also present in many smooth muscles but their function remains to be fully characterised. As with voltage-gated Ca^{2+} channels, these channel exist in three principle states; the resting, open and inactivated states. Voltage-gated Na^+ channels are characterised by their extremely fast activation and inactivation gating kinetics.

Structure and Pharmacology

The basic structure of voltage-gated sodium channels is similar to that of voltage-gated Ca^{2+} channels: The Na^+ -selective pore and voltage-sensor are both contained within an α -subunit which consists of four internally homologous domains each containing six transmembrane segments (Marban *et al.*, 1998). The alpha subunit is associated with two β -subunits, which enhance channel function by increasing peak amplitude, accelerating activation and inactivation gating and producing hyperpolarising shifts in the voltage-dependence of inactivation. As with Ca^{2+} channels, activity is further influenced by phosphorylation by PKA and PKC (producing current inhibition) (Marban *et al.*, 1998). Voltage-gated Na^+ channels are selectively blocked by neurotoxins such as tetrodotoxin (TTX) and saxitoxin (STX).

1.3.4. MYOMETRIAL Na^+ CURRENTS

The presence of voltage-gated Na^+ currents in pregnant rat myometrium was not readily apparent from early sucrose gap experiments although it was implied by the effects of removing external Na^+ (Kao & McCullough, 1975). This ambiguity was probably due to the difficulty in resolving very fast currents under conditions of very high capacitance and series resistance. As the techniques improved, fast Na^+ currents began to be reported. This current was described as a fast component of the inward current that was blocked when external Na^+ was removed and when a low concentration of tetrodotoxin (TTX) was added, but remained when the slower Ca^{2+} current was blocked with Mn^{2+} (Ohya & Sperelakis, 1989; Miyoshi *et al.*, 1991; Inoue & Sperelakis, 1991; Yoshino *et al.*, 1997). The activation threshold and potential of peak amplitude of this current was similar to that of the Ca^{2+} current (Ohya & Sperelakis, 1989; Miyoshi *et al.*, 1991; Inoue & Sperelakis, 1991), but the half inactivation potential was more negative (-59 mV; Yoshino *et al.*, 1997). The speed of decay of the Na^+ current was very fast ($\tau = 0.5$ ms at +10 mV compared to 32 ms for L-type Ca^{2+} current (fast component at +10 mV) (Ohya &

Sperelakis, 1989; Yoshino *et al.*, 1997). The amplitude of this current in the late pregnant rat myometrium was low compared to that of the Ca^{2+} current.

The $\text{Na}_v2.3$ sodium channel, which is part of a gene family distinct from the channels predominating in brain and skeletal muscle, has been immunolocalised to mouse uterine smooth muscle, and is co-localised with the gap junction protein, connexin 43 (Knittle *et al.*, 1996). It was proposed that this channel may either be involved in the spread of depolarisation through the tissue, hence the co-localisation with gap junctions, or it may act to inhibit or even reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange thus elevating cytoplasmic Ca^{2+} (Savineau *et al.*, 1987; Knittle *et al.*, 1996).

Despite evidence for mRNA expression of voltage-gated Na^+ channels in human uterus (George *et al.*, 1992; Boyle & Heslip, 1994), three studies have been unable to detect fast, TTX-sensitive Na^+ currents in freshly dispersed myocytes of human myometrium (Inoue *et al.*, 1990; Young *et al.*, 1991 & 1993) in contrast with the pregnant rat myometrium. However, in primary culture of pregnant human myometrium and a human uterine leiomyosarcoma cell line, a fast TTX-sensitive current with activation and inactivation characteristics of voltage-gated Na^+ current is present (Young & Herndon-Smith, 1991; Kusaka & Sperelakis, 1996). It is likely that these differences reflect changes in channel expression occurring during the culture process since in the cultured cells no evidence for T-type Ca^{2+} current was provided, even though it is the predominant inward current in freshly dispersed pregnant human myometrial cells (Inoue *et al.*, 1990; Young *et al.*, 1991 & 1993).

1.3.5. GESTATIONAL EFFECTS ON MYOMETRIAL Na^+ CURRENTS

Whole tissue expression of $\text{Na}_v2.3$ mRNA increases during pregnancy in mouse (Knittle *et al.*, 1996) and there is a similar rise in overall I_{Na} density (Inoue & Sperelakis, 1991). Yoshino *et al.* (1997) agreed that the occurrence of I_{Na} in rat myometrium increased steadily during gestation until at term it was the dominant current. However, they found that the current density increased during the first trimester of pregnancy, decreased in the second trimester and then increased by up to 7-fold between days 17 and 21, compared to day 0. The density of I_{Na} declined dramatically post-partum.

1.3.6. NONSELECTIVE CATION AND CHLORIDE CURRENTS

Many agonists including oxytocin, and some prostaglandins, stimulate Ca^{2+} influx through G-protein dependent receptor binding (Nebigil & Malik, 1993). OT induces at least two slow inward currents when the cell membrane potential is clamped at or near the resting level (Shimamura *et al.*, 1994b; Arnaudeau *et al.*, 1994). The first requires external Ca^{2+} but is not blocked by nifedipine or Mn^{2+} (Shimamura *et al.*, 1994b). The second is affected by changing Na^+ and Cl^- levels inside and outside the cell, is not induced by OT when EGTA is perfused into the cell (thus requiring intracellular Ca^{2+}), but is not blocked by isradipine or Cd^{2+} (Arnaudeau *et al.*, 1994). These two currents probably represent a calcium-activated- Cl^- current ($I_{\text{Cl,Ca}}$) (Coleman & Parkington, 1987) and a non-selective cation current (carried by Na^+ and Ca^{2+}), respectively. The resultant Na^+ influx and Cl^- efflux depolarises the cell, thus opening voltage-gated Ca^{2+} channels and contributing to the plateau-type action potential often observed in the myometrium (Coleman & Parkington, 1987). A large conductance Cl^- current has also been identified at the single channel level in pregnant guinea-pig and rat myometrium (Coleman & Parkington, 1987; Wang *et al.*, 1998).

Even though OT induces contractions and elevations of intracellular Ca^{2+} in rat and human myometrial which are inhibited by Ca^{2+} antagonists, (Batra, 1986; Kawarabayashi *et al.*, 1986b; Tasaka *et al.*, 1991; Thornton *et al.*, 1992a), a direct action of OT on Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels, as suggested in the pig by ZhuGe *et al.* (1995), is unlikely because OT also produces contractions in rat, guinea-pig and human myometrium under prolonged depolarisation with high K^+ (Kawarabayashi *et al.*, 1986b; Thornton *et al.*, 1992a) or in nominally Ca^{2+} -free solution (Coleman, McShane & Parkington, 1988; Chien *et al.*, 1996) (see below). The non-selective channel is probably activated by receptor gating to a G protein, whereas the $I_{\text{Cl,Ca}}$ current is probably activated indirectly via elevation in intracellular Ca^{2+} .

1.4. POTASSIUM CURRENTS

K^+ channels are present in all excitable tissues contributing to the control of membrane potential (as described earlier in this chapter) and in smooth muscle are essential for maintaining the tissue in a quiescent state, thus preventing or inhibiting contraction. Outward currents in myometrium were first identified as being carried by K^+ ions using the sucrose gap method in guinea-pigs and rats (Vasort, 1975; Kao & McCullough, 1975). This current was later found to comprise multiple components which varied in their characteristics between species and gestational or hormonal status (Mironneau & Savineau, 1980; Toro *et al.*, 1990a; Miyoshi *et al.*, 1991) or between experimental conditions. Many of these K^+ currents remain to be fully characterised.

1.4.1. VOLTAGE-GATED K^+ CHANNELS

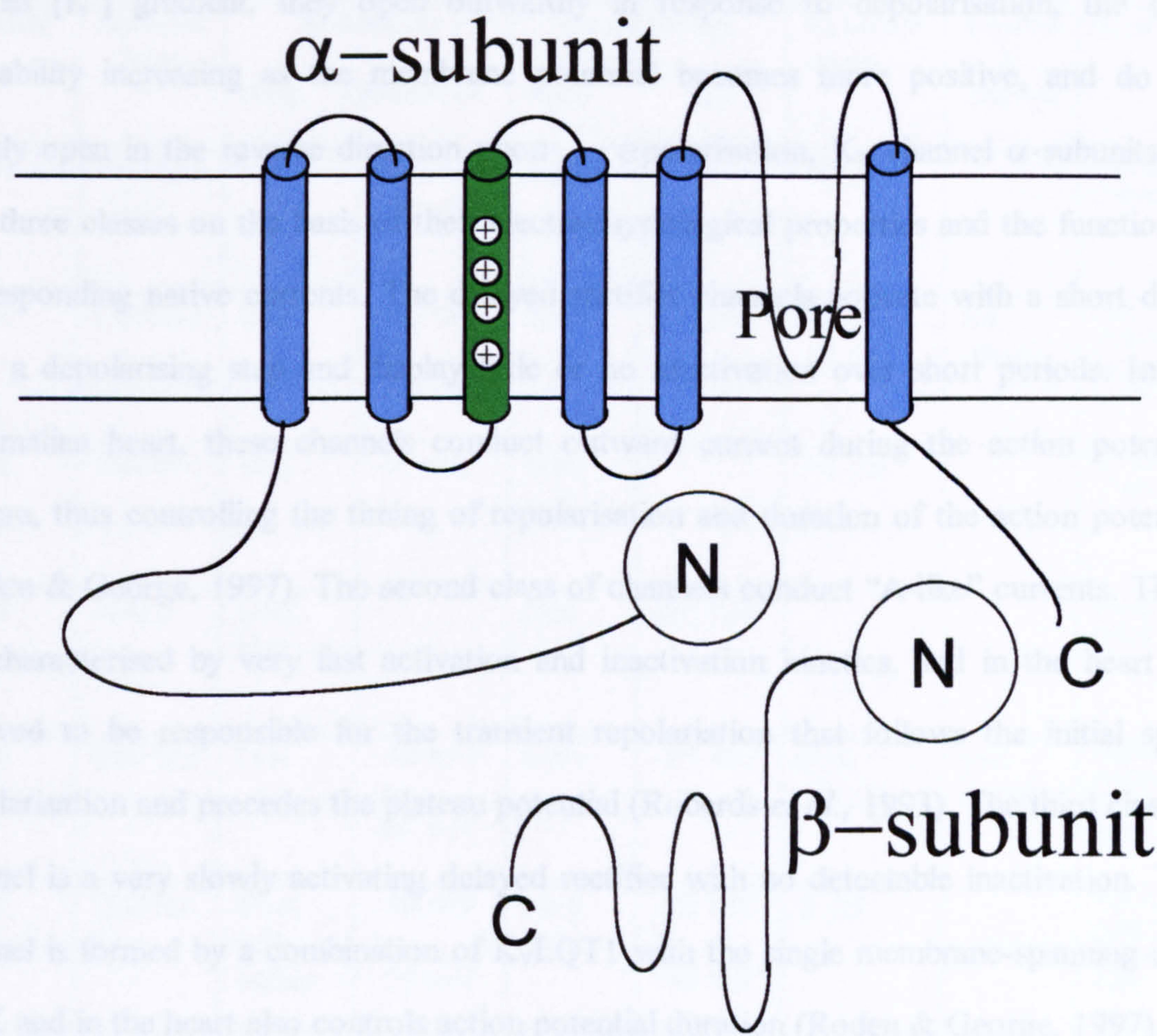
Basic structure


Voltage-gated K^+ channels (K_v channels) are the most extensive and diverse group of ion channels. There are at least four sub-families of functional mammalian K_v channels. These

include the K_v1, K_v2, K_v3 and K_v4 families, corresponding to the four *Drosophila* *shaker*, *shab*, *shaw* and *shal* genes, respectively. All of these channels share a common structure of six membrane-spanning domains, resembling one of the four repeated domains of the Na⁺ and Ca²⁺ α-subunits (Catterall, 1995; Deal *et al.*, 1996) (Fig 1.4.). Because of this similarity, these K⁺ channels are likely to be tetrameric in structure (MacKinnon, 1991). As with voltage-gated Na⁺ and Ca²⁺ channels, all K_v channels contain a voltage-sensor which, in response to depolarisation, produces a conformational change allowing the channel pore to open. Inactivation of K_v channels is also voltage-dependent and occurs through at least two mechanisms, one called N-type inactivation, where the cytoplasmic face of the pore is occluded by a ball-like peptide at the amino-terminus of the channel subunit, and the other C-type inactivation, which involves closing of the pore itself (Rasmussen *et al.*, 1998).

In addition to homotetrameric channels, K_v α-subunits are capable of forming heterotetrameric channels composed of different members of the same α-subunits family, with gating and pharmacological properties distinct from the sum of the properties of the two component subunits (Roberds *et al.*, 1993). This diversity is complicated further firstly by the existence of at least 3 types of β-subunit, which combine with α-subunit tetramers (see Fig 1.4.) and alter their activation and inactivation properties in a similar manner to β-subunits of Na⁺ and Ca²⁺ channels (Kukuljan *et al.*, 1995; Deal *et al.*, 1996), and secondly by at least four additional families of α-subunit, which in themselves are electrically silent but, in combination with K_v1, 2, 3 and 4 α-subunits, produce channels with unique properties (Salinas *et al.*, 1997).

FIG 1.4. STRUCTURE OF VOLTAGE-GATED K⁺ CHANNELS



	Voltage-sensor
C	Carboxy-terminus
(N)	Amino-terminus "ball peptide"

Diversity of kinetics and voltage-sensitivity

All K_v channels demonstrate outward rectification, that is, from the resting state, under a normal $[K^+]$ gradient, they open outwardly in response to depolarisation, the open probability increasing as the membrane potential becomes more positive, and do not readily open in the reverse direction upon repolarisation. K_v channel α -subunits fall into three classes on the basis of their electrophysiological properties and the function of corresponding native currents. The delayed rectifier channels activate with a short delay after a depolarising step and display little or no inactivation over short periods. In the mammalian heart, these channels conduct outward current during the action potential plateau, thus controlling the timing of repolarisation and duration of the action potential (Roden & George, 1997). The second class of channels conduct “A-like” currents. These are characterised by very fast activation and inactivation kinetics, and in the heart are believed to be responsible for the transient repolarisation that follows the initial spike depolarisation and precedes the plateau potential (Roberds *et al.*, 1993). The third class of channel is a very slowly activating delayed rectifier with no detectable inactivation. This channel is formed by a combination of K_vLQT1 with the single membrane-spanning unit, *minK* and in the heart also controls action potential duration (Roden & George, 1997). As with voltage-gated Ca^{2+} and Na^+ channels, K_v channels cycle between at least three states in response to membrane potential changes (Fig 1.2.). Most voltage-gated K^+ channels demonstrate strong outward rectification. This means that they are more likely to open at membrane potentials positive of the reversal potential, thus conducting hyperpolarising outward currents, than at membrane potentials negative of the reversal potential, where they would otherwise conduct depolarising inward current. Some of the cloned K_v channels and their electrophysiological properties are listed in table 1.2.

TABLE 1.2. ELECTROPHYSIOLOGICAL PROPERTIES OF SOME CLONED MAMMALIAN K_v CHANNELS

Channel	Current Type	Activation		Inactivation		Conduct- ance	Found in uterus	Authors
		Rate	Threshold	Rate	V _{0.5}			
K _v 1.1	DelRec	fast	below -60mV	slow		10 pS		Grissmer (1994)
K _v 1.2	DelRec	fast	-40 mV or -60 mV	slow	-15 mV	14 or 18 pS	no (dog)	Hart (1993) Grissmer (1994)
K _v 1.3	DelRec	fast	-60 mV	slow		14 pS		Grissmer (1994)
K _v 1.4	A-like	very fast		fast	-66 mV			Roberds (1993)
K _v 1.5	DelRec	fast	-40 mV or -60 mV	slow	-21 mV	8 or 10 pS	yes (dog)	Overturf (1994) Grissmer (1994)
K _v 2.1	DelRec	slow with delay	-30 mV to -50 mV	very slow	-25 mV to -45 mV	9 pS		Pak,1991a Patel,1997 Klemic, 1998
K _v 2.2	DelRec	slow with delay	-20 mV	very slow	-16 mV	15 pS	yes (dog)	Schmalz (1998)
K _v 3.1	DelRec	fast	-20 mV	slow		27 pS		Grissmer (1994)
K _v 4.1 (mShal)	A-like	very fast	-50 mV	fast	-69 mV			Pak (1991b)
K _v LQT1 <i>I_{min}K</i>	very slow DelRec	very slow	-30 mV	very very slow	—		yes (rat)	Boyle (1987a)

DelRec = delayed rectifier

TABLE 1.3. PHARMACOLOGICAL PROPERTIES OF SOME CLONED MAMMALIAN K_v CHANNELS

Channel	Current Type	Inhibitor (EC ₅₀)			Author(s)
		4-AP	TEA	Clofilium	
K _v 1.1.	fast delrec	290 µM	0.3µM (mouse) 20 mM (human)	>20 µM	Grissmer, 1994 Suessbrich, 1997
K _v 1.2.	fast delrec	74 - 590 µM	>100 mM	174 µM	Grissmer, 1994 Hart, 1993 Yamagishi, 1995
K _v 1.3.	fast delrec	200 - 300 µM (human)	10 mM (mouse) 30 mM (human) 50 mM (rat)	60 µM	Attali, 1992 Grissmer, 1994
K _v 1.4.	A-like	700 µM	>50 mM	> 1mM	Roberds, 1993 Yamagishi, 1995
K _v 1.5.	fast delrec	50 µM (human) 270 µM (dog)	>100 mM	0.15 -1 µM (human), 50 µM (dog)	Overturf, 1994 Grissmer, 1994 Fedida, 1993 Deal, 1996 Malayev, 1995
K _v 2.1.	slow delrec	>20 mM	5 mM		Pak, 1991a Kirsch, 1993
K _v 2.2.	slow derec	voltage-dependent	3 mM		Schmalz, 1998
K _v 3.1.	fast delrec	30 - 100 µM	0.2 mM		Grissmer, 1994 Kirsch, 1993
K _v 4.1. (mShal)	A-like	>2 mM	insensitive		Pak, 1991b
K _v 4.2.	A-like	1.5 mM	15 mM	>20 µM	Deal, 1996 Suessbrich, 1997
minK/ K _v LQT1	very slow delrec	>>3 mM	20 mM	80 µM	Attali, 1992

DelRec = delayed rectifier

Most K_v channels are also blocked by high concentrations of Ca²⁺ channel antagonists nifedipine, verapamil and diltiazem and partially blocked by high (µM) concentrations of charybdotoxin (Grissmer *et al.*, 1994).

Pharmacology of K_v Channels

K_v channels are sensitive to block by a wide range of pharmacological agents, the potency of which varies considerably between channel types. The effects of some of these drugs, which include tetraethylammonium (TEA), 4-aminopyridine (4-AP), charybdotoxin (ChTX), iberiotoxin (IbTX) and clofilium (Clf), on some of the cloned K_v channels, are listed in Table 1.3.

1.4.2. MYOMETRIAL K_v CURRENTS

The existence of voltage-gated K⁺ currents in myometrium was first indicated by the effect of the K⁺ channel blocker 4-AP, which inhibited both transient and sustained components of the outward K⁺ current in guinea-pig and rat myometrium and their insensitivity to low concentrations of TEA (Vasort, 1975; Mironneau & Savineau, 1980; Mironneau *et al.*, 1981; Miyoshi *et al.*, 1991). This current often had transient and sustained components, the occurrence of which, as well as 4-AP sensitivity, often varied between studies.

Transient or “A-like” K⁺ Currents

Early studies describe a transient component of the K⁺ current in myometrium of pregnant and non-pregnant rats, immature rats, non-pregnant guinea-pigs and non-pregnant humans which was completely blocked by 1-10 mM 4-AP but not 2-20 mM TEA and was activated by hyperpolarising conditioning pre-potentials or by holding potentials negative of -70 mV (Vasort, 1975; Mironneau & Savineau, 1980; Mironneau *et al.*, 1981; Miyoshi *et al.*, 1991; Piedras-Renteria, 1991; Erulkar *et al.*, 1993; Inoue *et al.*, 1993; Erulkar *et al.*, 1994; Wang *et al.*, 1998).

Electrophysiological analysis of this current confirmed that it inactivated in a voltage-dependent manner but calculations of half-inactivation ($V_{0.5}$) vary considerably between studies (eg -41 mV, Miyoshi *et al.*, 1991; -24 mV, Erulkar *et al.*, 1993; -48 mV, Erulkar *et*

al., 1994; -77 mV, Wang *et al.*, 1998). Because of its fast activation, fast inactivation and relatively negative threshold of activation (between -60 and -40 mV) this current often overlapped with inward calcium currents (Mironneau *et al.*, 1981; Erulkar *et al.*, 1993; Erulkar *et al.*, 1994; Inoue *et al.*, 1993; Wang *et al.*, 1998).

The differences in the voltage-sensitivity of inactivation of the A-current in the different studies may be due ^{to} the different concentrations of external Ca^{2+} used. There is some evidence to suggest that the A-like current in myometrium is Ca^{2+} -sensitive and Wang *et al.* (1998) demonstrated that raising the external $[\text{Ca}^{2+}]$ from 1 to 30 mM overcame the inactivation of the current at a holding potential of -50 mV (hence shifting the inactivation threshold to the right). Although Vasort (1975) found that the transient current was inhibited by D-600 and Mn^{2+} , implicating modulation by $[\text{Ca}^{2+}]_i$, Piedras-Renteria *et al.* (1991) showed that it was not inhibited by putting EGTA in the pipette solution, suggesting that it was sensitive to external $[\text{Ca}^{2+}]$ and not $[\text{Ca}^{2+}]_i$.

Abundance of the A-like current appeared to be dependent on gestational or hormonal status. For example, in leiomyoma cells of human myometrium it was more abundant than in normal non-pregnant tissue (Erulkar *et al.*, 1993). Similarly, it was frequently present in myometrium of adult non-pregnant rat (50 % of cells) but absent in late-pregnant rat (Wang *et al.*, 1998). In immature rat myometrium this current occurred in the majority of cells (79 % of cells) but was inhibited by estrogen treatment (30% of cells) (Erulkar *et al.*, 1994).

“Delayed Rectifier”-Like K^+ Currents

In addition to the transient component of the K^+ current, there is a sustained or slowly decaying component which in early studies in rat and guinea-pig myometrium was inhibited by 4-AP with a comparable potency to that demonstrated against the transient component, but, unlike the transient current, it was also partially sensitive to TEA (Vasort,

1975; Mironneau & Savineau, 1980; Toro *et al.*, 1990a; Miyoshi *et al.*, 1991). A similar current was then found in cultured non-pregnant human myometrium, characterised by partial block with 4-AP (1 mM), partial block with 2 mM TEA, slow decay and threshold of activation near -40 mV (Erulkar *et al.*, 1993). In immature rat myometrium by contrast, a 4-AP sensitive component of the sustained K^+ current was not present (Erulkar *et al.*, 1994). To add to this, in pregnant rat myometrium the existence of an (at that time) uncharacterised K^+ current was implied by the incomplete inhibition with high concentrations of either 4-AP (10 mM) or TEA (50 mM) (Inoue *et al.*, 1993). A major criticism of these earlier studies is therefore that detailed electrophysiological characterisation of the sustained current (ie voltage-dependence of inactivation) was not performed and care was not taken to isolate it (or them) from the Ca^{2+} -activated K^+ current.

However, in a recent study in pregnant and non-pregnant rat myometrium, a very detailed electrophysiological and pharmacologic description of these currents was presented (Wang *et al.*, 1998). The slowly decaying component of the K^+ current was found to be reduced by 50 % in non-pregnant and by 70 % in pregnant myometrium when the holding potential was changed from -80 mV to -50 mV, suggesting voltage-dependence of inactivation. In both pregnant and non-pregnant myometrium, this inactivation was found to be biphasic suggesting the existence of two voltage-gated K^+ currents, distinct from the A-like current, one with a $V_{0.5}$ of around -60 mV (called C_1), the other with a $V_{0.5}$ of around -20 mV (called C_2). C_1 also had a negative threshold of activation (below -60 mV). Pharmacologically, C_1 was partially blocked by TEA, but only weakly sensitive to ChTX, IbTX and 4-AP, whereas C_2 was partially blocked by TEA, ChTX but more sensitive to 4-AP (EC_{50} 1 mM).

Another very slowly activating and slowly decaying K^+ current is also present in rat myometrium, which demonstrates voltage-dependence of activation and is upregulated

under conditions where estrogen levels are higher (Boyle *et al.*, 1987a; Boyle *et al.*, 1987b). This current is similar to that described for the cloned K_vLQT1 channel from rat uterus and heart (Folander *et al.*, 1990).

Single Channel K_v Currents

The single channel conductances of myometrial K_v currents are largely unexplored. In addition to the large conductance BK_{Ca} channel (see below), two smaller conductances (~20 pS and ~50 pS) were reported in pregnant rat myometrium and cultured human myometrium (Wang *et al.*, 1998; Anwer *et al.*, 1993), but apart from confirming K⁺-selectivity, the pharmacological and electrophysiological properties of these channels were not investigated further.

1.4.3. LARGE CONDUCTANCE, Ca²⁺-ACTIVATED K⁺ CHANNELS

Large conductance Ca²⁺-activated channels (BK_{Ca} or *maxiK*) are characterised by a large conductance (up to 280 pS; Wallner *et al.*, 1995) and a dependency on membrane potential and intracellular calcium for channel opening. As with the voltage-gated K⁺ channels, BK_{Ca} channels prefer outward rectification, conducting outward current upon depolarisation rather than inward current in response to hyperpolarisation.

Basic Structure

BK_{Ca} channels share the basic structure of K_v channels, the pore-forming α -subunit having six transmembrane domains, including a voltage-sensor and a distinct pore domain. In addition they have a large carboxyterminal domain, possibly responsible for the Ca²⁺-dependency (Wei *et al.*, 1994). BK_{Ca} channels in mammalian smooth muscle are coded from a single family of *Slo* genes and mRNA for h*Slo* has been cloned from human myometrium (Wallner *et al.*, 1995). In smooth muscle, BK_{Ca} α -subunit function is

modulated by association with a β -subunit which enhances channel Ca^{2+} sensitivity, causing a hyperpolarising shift in the threshold of activation (Tanaka *et al.*, 1997).

Pharmacology

BK_{Ca} channels are blocked by TEA^{*} ($\text{EC}_{50} \sim 250 \mu\text{M}$), IbTX ($\text{EC}_{50} < 20 \text{ nM}$), ChTX ($\text{EC}_{50} < 40 \text{ nM}$), paxilline (effect dependent on $[\text{Ca}^{2+}]$), and intracellular Ba^{2+} , and are insensitive to 4-AP, apamin and the ATP-sensitive K^+ channel opener lemakalim (Wallner *et al.*, 1995; McCobb *et al.*, 1995).

1.4.4. MYOMETRIAL B_{K,ca} AND B_K CURRENTS

Whole Cell Currents

Early sucrose gap studies in non-pregnant guinea-pig and pregnant rat indicated that a component of the outward current was reduced by lowering external $[\text{Ca}^{2+}]$ or the addition of Ca^{2+} channel antagonists, as well as TEA, and was increased by increasing external $[\text{Ca}^{2+}]$ and was either transient (Vasort, 1975) or sustained (Mironneau & Savineau, 1980). The dependence on Ca^{2+} and sensitivity to TEA (EC_{50} of 0.1 to 1mM) but not 4-AP was later confirmed by single cell patch clamp in pregnant human and pregnant and non-pregnant rat myometrium (Inoue *et al.*, 1990; Piedras-Renteria *et al.*, 1991; Anwer *et al.*, 1993; Wang *et al.*, 1998). Apamin, the selective blocker of small conductance Ca^{2+} -activated K^+ current had little or no effect on K^+ current in pregnant rat myometrial cells (Wang *et al.*, 1998).

Single Channel Currents

The large conductance Ca^{2+} -activated K^+ channel (BK_{Ca}) is the most frequently recorded K^+ conductance in single channel studies, with conductances ranging between 140 pS and 260 pS in myometrium of non-pregnant and pregnant rats, non-pregnant and pregnant humans and an immortalised human myometrial cell line (Toro *et al.*, 1990b; Perez *et al.*,

*external application.

1993; Anwer *et al.*, 1993; Khan *et al.*, 1993; Perez & Toro, 1994; Meera *et al.*, 1995; Khan *et al.*, 1997; Wang *et al.*, 1998). By increasing $[Ca^{2+}]$ on the cytoplasmic side of the patch from 10-100 nM (close to the physiological concentration) to 10-100 μ M, the membrane potential dependence of activation of this channel is shifted in the negative direction by up to 50 mV, due to an increased channel open probability (Toro *et al.*, 1990b; Khan *et al.*, 1993; Perez *et al.*, 1993; Khan *et al.*, 1997). $I_{K(Ca)}$ in the myometrium is also blocked by cytoplasmic application of micromolar Ba^{2+} and TEA and external application of TEA and charybdotoxin (Khan *et al.*, 1993; Perez *et al.*, 1993; Khan *et al.*, 1997).

Intracellular Modulation of BK_{Ca}

BK_{Ca} is modulated by phosphorylation via protein kinases such as PKA and by G protein binding. In rat myometrial lipid bilayers $I_{K(Ca)}$ is activated by GTP or GTP γ S in the presence of Mg^{2+} , an effect which is potentiated by the β -agonist isoprenaline, suggesting direct activation of the channel by a G protein (Toro *et al.*, 1990b). The effect of PKA on $I_{K(Ca)}$ depends on gestational or hormonal status, channel open probability being reduced by PKA in non-pregnant rat myometrium and enhanced in pregnant rat and human myometrium (Perez & Toro, 1994). PKA-dependent activation of BK_{Ca} is stimulated by the uterine relaxant relaxin (Meera *et al.*, 1995).

In human myometrium from women in labour, Khan *et al.* (1993 & 1997) found that BK_{Ca} is replaced by BK, a channel of similar conductance but which was insensitive to $[Ca^{2+}]_i$ and was not voltage-dependent (ie was open with equal probability at all potentials between -60 and +60 mV). This channel differs in its sensitivity to Ba^{2+} and TEA and, in cell-attached patch mode, exhibits either very sparse activity compared to inside-out patch mode or develops cyclical opening behaviour over a period of minutes, suggesting that it is being inhibited or modulated by intracellular factors.

1.4.5. INWARD RECTIFIERS AND ATP-SENSITIVE K⁺ CHANNELS

Inward rectifier channels (K_{IR}) are structurally dissimilar to K_V channels, having only two transmembrane segments and lacking a voltage-sensor component, enabling them to open at all potentials (Roden & George, 1997). For example, at potentials positive of the K⁺ reversal potential K_{IR} channels conduct outward (repolarising) current but at potentials negative of the reversal potential they conduct inward (depolarising) current. They do however demonstrate voltage-dependence but, in contrast to the K_V channels, the open probability decreases as the membrane potential becomes more positive, hence producing a negative outward rectification, which becomes an inward rectification when the reversal potential is shifted to zero by symmetrical internal and external [K⁺]. In cardiac muscle, this means that K_{IR} channels are able to set a negative resting membrane potential without interfering with action potential generation.

The pore forming subunits of K_{ATP} channels are structurally related to inward rectifiers (K_{IR}), but are weakly voltage-dependent, demonstrating linear current voltage relationships in symmetrical K⁺ solutions, or weakly outwardly rectifying current-voltage relationships in physiological K⁺ solutions (Quayle *et al.*, 1997). Association of the K_{IR} 6.1 subunit with the sulphonylurea receptor, a regulatory sub-unit of the K_{ATP} channel (Yamada *et al.*, 1997), confers sensitivity of the channel to intracellular ATP. Binding of ATP inactivates the channel and dissociation of ATP from this site activates it. K_{ATP} channels are therefore sensitive to the metabolic status of the cell, and may open during metabolic compromise, hence causing relaxation (Quayle *et al.*, 1997). K_{ATP} channels are inhibited by sulphonylurea drugs such as glibenclamide (Quayle *et al.*, 1997).

1.4.6. MYOMETRIAL K_{IR} AND K_{ATP} CURRENTS

Hyperpolarisation in guinea-pig myometrium, under voltage-clamp conditions, induces a slow inward current (Parkington and Coleman, 1988) which may correspond to the inward

rectification recorded in intestinal smooth muscle (Benham *et al.*, 1987). The presence of K_{ATP} current in myometrium is implied by the relaxant effects of drugs that open K_{ATP} channels, such as chromakalim, aprikalim and levchromakalim on contracting myometrium and their antagonism by glibenclamide (Piper *et al.*, 1990; Cheuk *et al.*, 1993; Morrison *et al.*, 1993). Glibenclamide also partially (50%) inhibits cyanide-induced ^{86}Rb efflux in strips of pregnant rat myometrium (Heaton *et al.*, 1993). These effects suggest that K_{ATP} channels, along with BK_{Ca} and K_V channels contribute to setting the resting membrane potential in myometrium and may contribute to relaxation in response to metabolic changes. Direct measurements of K_{IR} or K_{ATP} currents in myometrium, either at the whole cell or single channel level, however, remain to be performed.

1.4.7. Na^+/K^+ ATPase

The Na^+/K^+ ATPase is electrogenic, exchanging 3K^+ (out) for 2Na^+ (in) thus hyperpolarising the membrane. This pump probably contributes to the resting membrane potential in myometrium since inhibition of the pump by ouabain (a selective blocker of the Na^+/K^+ ATPase) caused depolarisation and hence activation of voltage-gated Ca^{2+} channels, and in the rat this resulted in regular but short-lived phasic contractions which were inhibited by nifedipine (Ausina *et al.*, 1996). There is also evidence in the pregnant human myometrium that the Na^+/K^+ ATPase is responsible for the large hyperpolarisation that follows the plateau-type action potentials induced by PGE_2 and $\text{PGF}_{2\alpha}$ (Parkington *et al.*, 1999).

1.5. RELATIONSHIP BETWEEN Ca^{2+} -INFLUX, K^{+} -EFFLUX AND EXCITATION-CONTRACTION COUPLING

1.5.1. EFFECT OF Ca^{2+} -CHANNEL ANTAGONISTS ON MYOMETRIAL CONTRACTION

It is now established that spontaneous contractions are always preceded by action potentials and that the depolarisation requires Ca^{2+} influx (Mironneau, 1973; Amedee *et al.*, 1986a). In all smooth muscles, an increase in free intracellular Ca^{2+} is essential for both depolarisation-induced and agonist-induced contractions (Himpens & Somlyo, 1988), acting via the calmodulin/myosin light chain kinase pathway (Horowitz *et al.*, 1996). Removing external Ca^{2+} abolishes spontaneous contractions of pregnant rat and human myometrium *in vitro*, whereas elevating the Ca^{2+} concentration increases contraction amplitude (Granger *et al.*, 1986; Ballejo *et al.*, 1986; Poli *et al.*, 1990). Furthermore, a wide range of L-type Ca^{2+} channel-selective antagonists, as well as micro- or milli-molar concentrations of divalent cations such as Co^{2+} , Cd^{2+} and Mn^{2+} (Sipowicz *et al.*, 1995; Mogami & Todoroki, 1997), inhibit spontaneous and agonist-induced contractions of the rat, sheep and human myometrium *in vitro* (see Table 1.4.). Ca^{2+} channel antagonists also shift Ca^{2+} -contraction dose responses to the right (Granger *et al.*, 1986; Ballejo *et al.*, 1986), proving the functional coupling of contraction with calcium influx through voltage-gated channels. *In vivo*, Ca^{2+} channel antagonists prevent spontaneous and OT or $\text{PGF}_{2\alpha}$ -induced labour in rats (Csapo *et al.*, 1982), inhibit contractions of the non-pregnant rat uterus (Abel & Hollingsworth, 1985), but have variable or limited effects on uterine contraction in pregnant women in labour (Higby *et al.*, 1993). The blocker of voltage-gated Na^{+} channels, TTX ($1\mu\text{M}$) is ineffective at inhibiting oxytocin-induced contractions of pregnant rat myometrium and spontaneous contractions of pregnant human myometrium *in vitro* (Poli *et al.*, 1990; Phillippe & Basa, 1997a; Parkington *et al.*, 1999).

TABLE 1.4.

Inhibition of Myometrial Contraction <i>in vitro</i> by Ca ²⁺ Channel Antagonists				
Species	Preparation	Contraction	Drug (dose*)	Author(s)
Rat	pregnant single cells, Ca ²⁺ 2 mM	Spontaneous & K ⁺ -induced	D-600 (1 µM)	Amedee <i>et al.</i> 1986a
Rat	Non-preg strips, Ca ²⁺ 3 mM	K ⁺ or ACh & 5-HT-induced	verapamil (1-10 µM), diltiazem (1-10 µM)	Ichida <i>et al.</i> 1984
Rat	Pregnant strips Ca ²⁺ 1.3 mM	Spontaneous or K ⁺ -induced	gallopamil (100 nM), diltiazem (0.5-1 µM), nifedipine (10 nM), cinnarizine (10nM-5 µM)	Granger <i>et al.</i> , 1986
Rat	Non-preg strips Ca ²⁺ 1.8 mM	BAYK-8644-induced	nifedipine (1-10 µM)	Chien <i>et al.</i> , 1996
Rat	Non-preg strips	Oxytocin-induced	nifedipine (1 µM)	Phillippe & Basa, 1997a
Rat	post-partum strips	spontaneous	nicardipine (100µg/ml)	Csapo <i>et al.</i> , 1982
Sheep	pregnant strips, Ca ²⁺ 2.5 mM	electrically-induced	verapamil (1 µM)	Parkington, 1985
Human	pregnant strips	spontaneous & K ⁺ , PGF _{2α} , OT & VP- induced	nifedipine, nitrendipine	Maigaard <i>et al.</i> , 1983
Human	pregnant strips, Ca ²⁺ 2.5 mM	spontaneous & OT-induced	diltiazem (10 ⁻⁶ g/ml) & (10 ⁻⁴ g/ml)	Kawarabayashi <i>et al.</i> , 1986b
Human	pregnant strips, Ca ²⁺ 2.5 mM	spontaneous & K ⁺ -induced	nifedipine (0.01-1 µM), verapamil (1-10 µM), cinnarizine (100 µM)	Ballejo <i>et al.</i> , 1986
Human	pregnant strips, Ca ²⁺ 1.9 mM	spontaneous	nifedipine (0.1-1 nM), verapamil (10-100nM), diltiazem (1-10 µM)	Poli <i>et al.</i> , 1990
Human	pregnant strips, Ca ²⁺ 2.5 mM	spontaneous & PG-induced	nifedipine (1 µM) verapamil (10 µM)	Parkington <i>et al.</i> , 1999

* concentration producing 50-100% inhibition of contraction. ACh= acetylcholine, 5-HT= seretonin, VP= vasopressin, OT=oxytocin.

The importance of Ca^{2+} influx to myometrial contraction is also illustrated by the actions of the dihydropyridine derivative, BAYK-8644, an L-type Ca^{2+} channel agonist, which induces or enhances phasic contractions in isolated strips of pregnant rat and human myometrium (Poli *et al.*, 1989; Chien *et al.*, 1996; Phillippe & Basa, 1996; Kyojuka *et al.*, 1987), antagonises the inhibitory action of nifedipine (Poli *et al.*, 1989; Poli *et al.*, 1990) and shifts the concentration-dependency of contraction to K^+ to the left (Kyojuka *et al.*, 1987).

1.5.2. EFFECTS OF K^+ CHANNEL BLOCKERS AND OPENERS ON MYOMETRIAL CONTRACTION

There is also functional evidence for the importance of K^+ currents in the prevention or suppression of spontaneous myometrial contractions. The selective inhibitor of BK_{Ca} channels, iberiotoxin (IbTX, 50 nM) induces spontaneous contractions in quiescent strips of pregnant human myometrium (Anwer *et al.*, 1993). This effect is probably via depolarisation and subsequent opening of voltage-gated Ca^{2+} channels and elevation of $[\text{Ca}^{2+}]_{\text{i}}$. IbTX (40 nM) and TEA (5 mM) also increase the frequency and force of spontaneous contractions of non-pregnant rat myometrium *in vitro* (Anwer *et al.*, 1993; Taggart & Wray, 1998). In pregnant human myometrial strips, both 4-AP (5 mM) and charybdotoxin (10 nM) prolonged $\text{PGF}_{2\alpha}$ and PGE_2 -induced contraction duration whereas 2 mM TEA increased the frequency of spontaneous contractions (Parkington *et al.*, 1999). In contrast, spontaneous and 10-20 mM KCl or OT-induced phasic contractions of pregnant rat and human myometrium have been inhibited by drugs that stimulate opening of K_{ATP} and/or BK_{Ca} channels (Piper *et al.*, 1990; Cheuk *et al.*, 1993; Morrison *et al.*, 1993; Khan *et al.*, 1998).

1.5.3. CONTROL OF INTRACELLULAR Ca^{2+} CONCENTRATION (SUMMARISED IN FIG 1.5.)

Intracellular Ca^{2+} stores

Although Ca^{2+} influx may be an essential trigger for contraction, an intracellular Ca^{2+} concentration sufficient for the activation of the contractile apparatus may also require release of stored Ca^{2+} . In human myometrium, the relative importance of Ca^{2+} stores is poorly understood, but much work has been performed in the rat.

In all smooth muscles intracellular Ca^{2+} is stored in the sarcoplasmic reticulum (SR) and its release is stimulated by at least two different mechanisms. The hydrolysis product of phospholipase C, inositol 1,4,5, trisphosphate (IP_3) is generated in response to G-protein coupled receptor stimulation (Berridge *et al.*, 1993) by such agonists as oxytocin. IP_3 binds to receptors on the IP_3 -sensitive Ca^{2+} store (ISCS) and triggers Ca^{2+} release. This binding is itself influenced by Ca^{2+} , which forms a positive feedback at less than 300 nM Ca^{2+} and a negative feedback at higher concentrations (Ino & Tsukioka, 1994). The second principle mechanism is Ca^{2+} -induced- Ca^{2+} -release (CICR) which involves stimulation of Ca^{2+} release from a Ca^{2+} -sensitive Ca^{2+} store (CSCS) through the opening of Ca^{2+} -gated channels, also known as ryanodine receptors (Lynn *et al.*, 1993; Giannini *et al.*, 1995). Ca^{2+} also feeds back negatively on the ryanodine receptor at higher concentrations. Ca^{2+} is pumped back into the SR through Ca^{2+} -ATPases (Wu *et al.*, 1995), although in the myometrium the relative importance of this pathway to myometrial contractility remains a matter of debate (Phillippe *et al.*, 1995b; Taggart & Wray, 1998; Tribe *et al.*, 1999).

Refilling of stores in myometrium appears to be dependent on extracellular Ca^{2+} influx (Thornton *et al.*, 1992a). In some cell types, this may occur directly from the extracellular Ca^{2+} pool without an intermediate rise in $[\text{Ca}^{2+}]_i$, through a mechanism called capacitative

Ca^{2+} entry (Horowitz *et al.*, 1996). Ca^{2+} may also be sequestered, either in the cytoplasm by endogenous buffer molecules, or in the mitochondria (Horowitz *et al.*, 1996).

Plasma Membrane Ca^{2+} Pumps and Transporters

As well as being controlled by internal stores, the intracellular Ca^{2+} concentration in the myometrium is also reduced by extrusion from the cell through a $\text{Ca}^{2+}/\text{H}^{+}$ ATPase pump and a $\text{Ca}^{2+}/\text{Na}^{+}$ transporter which exchanges 3Na^{+} for each Ca^{2+} and is bidirectional (Grover *et al.*, 1981; Ver *et al.* 1989; Morgan *et al.*, 1993a).

Calcium Oscillations

Oscillations in free intracellular Ca^{2+} concentration occur in many cell types, including smooth muscle (Berridge & Gallione, 1988). In a small percentage of cultured human myometrial cells *in vitro* (2-15%), spontaneous oscillations in intracellular Ca^{2+} occur (Thornton *et al.*, 1992b; Morgan *et al.*, 1993b; Lynn *et al.*, 1993). The mechanism(s) by which these oscillations occur or how they are related to phasic contractions in the myometrium are not fully understood, but as in other cell types they are believed to involve cyclic release and re-uptake of Ca^{2+} from both types of store, under the control of multiple positive and negative feedback loops as well as stimulus from IP_3 (see Fig 1.5.) and in rat myometrium are triggered by extracellular Ca^{2+} influx (Chien *et al.*, 1996).

Rhythmic contractions of the rat myometrium *in vitro* are induced or enhanced (increased frequency and amplitude) by drugs or hormones that induce IP_3 generation (eg OT; acetylcholine), or facilitate extracellular Ca^{2+} influx, either via opening of voltage-gated Ca^{2+} channels directly (eg BAYK 8644), or indirectly via depolarisation (eg 10-30 mM K^{+}) (Morrison *et al.*, 1993; Phillippe, 1994; Phillippe *et al.*, 1995a; Chien *et al.*, 1996; Phillippe & Basa, 1997b). These contractions are inhibited by agents that block IP_3 production, block Ca^{2+} -induced- Ca^{2+} -release, activate PKC and inhibit Ca^{2+} -influx through L-type Ca^{2+} channels (Phillippe, 1994; Chien *et al.*, 1996; Phillippe & Basa, 1996). In a more recent

study by contrast, cyclopiazonic acid (CPA), a blocker of the SR Ca^{2+} ATPase, *enhanced* contraction amplitude and duration or caused tetanic contractions, in myometrium from non-pregnant rats and pregnant rats, respectively (Taggart & Wray, 1998). Similarly, in human myometrium, CPA did not inhibit contractions or alter frequency but increased contraction duration in strips from non-labouring term pregnant women and caused tetany in those from women in labour at term (Tribe *et al.*, 1999). The relationship between rhythmic contractions and intracellular Ca^{2+} oscillations therefore requires further study (Fig 1.5.).

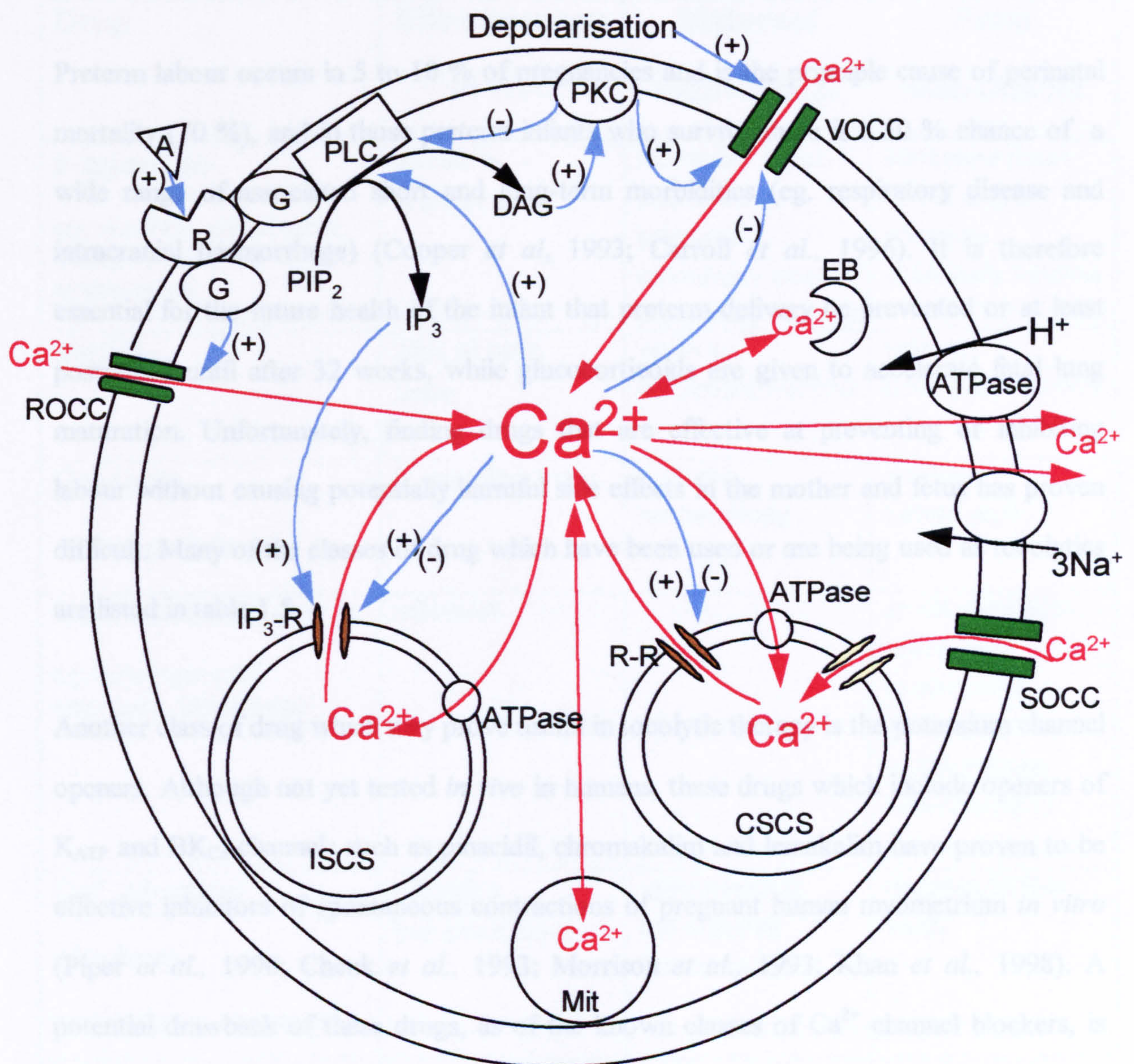
Effects of Uterotonins

Many agonists cause elevations in intracellular Ca^{2+} via multiple mechanisms. Oxytocin mobilises Ca^{2+} from internal stores via G protein linked receptors coupled to production of IP_3 , in guinea-pig, human, porcine and rat myometrium (Marc *et al.*, 1986; Schrey *et al.* 1987; Coleman, *et al.*, 1988; Anwer & Sanborn, 1989; Zhuge *et al.*, 1995) as well as stimulating extracellular Ca^{2+} influx. On continuous perfusion at low concentrations (1pM-1nM), OT induces intracellular Ca^{2+} oscillations in cultured human myometrial cells which are dependent on extracellular Ca^{2+} (Thornton *et al.*, 1992b), and in rat myometrial strips *in vitro* these OT-induced oscillations occur simultaneously with phasic contractions which are inhibited by L-type Ca^{2+} channel blockade (1 μM nifedipine) but not fast Na^+ channel blockade (1 μM TTX) (Phillippe & Basa, 1997a). In the cultured cells, each transient is preceded by a slow increase in baseline Ca^{2+} (similar in appearance to the pacemaker potentials or slow waves that precede action potentials) (Thornton *et al.*, 1992b).

The effects of prostaglandins on intracellular Ca^{2+} are complex. It is suggested by Thornton *et al.* (1992a) that unlike oxytocin, the PGE_2 is unable to stimulate $[\text{Ca}^{2+}]_i$ transients in the absence of extracellular Ca^{2+} in cultured human myometrium or in the presence of high external K^+ concentration, so is probably acting entirely on Ca^{2+} influx (probably via receptor-gated non-selective cation channels and subsequent

depolarisations). However, Asboth *et al.* (1996) have demonstrated, also in cultured human myometrium, that PGE₂ may also activate increase intracellular Ca²⁺ via IP₃ production. The response to PGF_{2α} also appears to involve multiple mechanisms. In non-pregnant and pregnant human myometrium it has raised [Ca²⁺]_i, an effect that was 50-100 % blocked by verapamil and was dependent on external Ca²⁺ (Molnar & Hertelendy, 1990; Parkington *et al.*, 1999). It has also caused contraction at least in part through release of stored Ca²⁺ but possibly via an IP₃ independent pathway (Schrey *et al.* 1987), either via an influence on Ca²⁺-induced Ca²⁺-release or cytoplasmic Ca²⁺ sequestration (Coleman *et al.*, 1988), or via a sensitization of the contractile proteins to Ca²⁺ (Izumi *et al.*, 1996).

FIG 1.5. REGULATION OF INTRACELLULAR Ca^{2+}



— stimulation (+) or inhibition (-)

— movement of Ca^{2+}

ISCS: IP_3 -sensitive Ca^{2+} store; CSCS Ca^{2+} -sensitive Ca^{2+} store; ROCC: receptor-operated Ca^{2+} channel; VOCC: voltage-operated Ca^{2+} channel; SOCC: store-operated Ca^{2+} channel; IP_3 -R: IP_3 receptor; R-R: ryanodine receptor; PIP_2 : phosphatidylinositol; IP_3 : inositol trisphosphate; DAG: diacylglycerol; PLC: phospholipase C; PKC: protein kinase C; G: G protein; R receptor; A: agonist; Mit: mitochondria; EB: endogenous buffer.

1.6. PREVENTION OF PRETERM LABOUR (TOCOLYSIS)

Preterm labour occurs in 5 to 10 % of pregnancies and is the principle cause of perinatal mortality (70 %), and in those preterm infants who survive there is a 50 % chance of a wide range of associated short and long-term morbidities (eg. respiratory disease and intracranial haemorrhage) (Cooper *et al.*, 1993; Carroll *et al.*, 1996). It is therefore essential for the future health of the infant that preterm delivery be prevented or at least postponed until after 32 weeks, while glucocorticoids are given to accelerate fetal lung maturation. Unfortunately, finding drugs that are effective at preventing or inhibiting labour without causing potentially harmful side effects in the mother and fetus has proven difficult. Many of the classes of drug which have been used or are being used as tocolytics are listed in table 1.5.

Another class of drug which may prove useful in tocolytic therapy is the potassium channel openers. Although not yet tested *in vivo* in humans, these drugs which include openers of K_{ATP} and BK_{Ca} channels such as pinacidil, chromakalim and lemakalim have proven to be effective inhibitors of spontaneous contractions of pregnant human myometrium *in vitro* (Piper *et al.*, 1990; Cheuk *et al.*, 1993; Morrison *et al.*, 1993; Khan *et al.*, 1998). A potential drawback of these drugs, as of the known classes of Ca^{2+} channel blockers, is that they would not be expected to be utero-specific, since they are also effective inhibitors of contraction in vascular smooth muscle (Quayle *et al.*, 1997). There are as yet no drugs known to selectively open voltage-gated K^+ channels. If voltage-gated K^+ channels specific to the uterus were found and if drugs were developed to selectively open these channels such drugs may prove useful and safe tocolytics.

TABLE 1.5. CLASSES OF TOCOLYTIC DRUG

Drug	Effectiveness <i>in vivo</i>	Maternal side-effects	Fetal side-effects
β-agonists eg. ritodrine, salbutamol, hexaprenaline, isoprenaline	variable & short-term	increased heart rate & blood pressure pulmonary edema potentially harmful	increased heart rate & blood pressure potentially harmful
MgSO₄	little at safe dose	various, potentially harmful	various, potentially harmful
Oxytocin antagonists	in ongoing trials	requires further study	requires further study
COX inhibitors eg. indomethacin, naproxen, fenoprofen	effective		<i>in late gestation:</i> necrotising enterocolitis, intracranial haemorrhage, non-closure of ductus arteriosus
Ca²⁺ channel blockers eg nifedipine, verapamil	effective but short term	vasodilatation but requires further study	requires further study
NO donors eg glyceryl trinitrate	requires further study	ripens cervix	requires further study

The information provided in this table is derived mostly from the reviews of Higby *et al.* (1993) and Lopez-Bernal *et al.* (1995).

1.7. AIMS OF THE STUDY

- 1). To characterise the electrophysiological and pharmacological properties of whole-cell voltage-gated Ca^{2+} channel currents in human myometrial smooth muscle cells (HMSMCs) from pregnant women.
- 2). To characterise the Ca^{2+} channel antagonist properties of the COX-2 selective cyclooxygenase inhibitor nimesulide in HMSMCs from pregnant women, with respect to its ability to inhibit spontaneous myometrial contractions *in vitro* and its potential for tocolysis.
- 3). To dissect the components of the whole cell outward K^{+} current in HMSMCs with respect to sensitivity to TEA and 4-AP and speed of decay (transient or sustained), and compare them in HMSMCs from pregnant and non-pregnant women.
- 4). To further characterise the transient 4-AP sensitive outward K^{+} current (A-current) and its relationship with inward Ca^{2+} current in HMSMCs.
- 5). To provide preliminary evidence for glibenclamide-sensitive K^{+} current in HMSMCs.
- 6). To provide further detailed electrophysiological and pharmacological characterisation of the slow decaying voltage-gated (delayed rectifier) K^{+} current(s) in HMSMCs from pregnant women.

Chapter 2

Electrophysiology Theory, Methods and Materials

2.1. TISSUE COLLECTION AND CELL ISOLATION

2.1.1. TISSUE COLLECTION

Approval for the use of human myometrial samples from St Thomas', and occasionally from Guy's or Queen Charlotte's Hospitals, London, was obtained from the respective Ethics Committees. Written, informed consent was obtained from patients prior to surgery. Myometrial biopsies were taken from the middle of the upper edge of the lower segment incision in pregnant women undergoing routine elective caesarean section at term but not in labour (38-40 weeks). The commonest reasons for caesarean section were breech presentation or the mother having had a previous elective section. Samples were also taken from non-pregnant women of pre-menopausal age (<45 years) at hysterectomy. Biopsies were collected, transported and stored in physiological saline solution (PSS, with 1.5 mM Ca^{2+} see 2.5.) at 4 °C . The samples were chopped into small 1-2 mm³ pieces and stored in PSS at 4 °C for up to 2 days prior to use. There was no evidence to indicate that storage for this period of time altered the amplitude or characteristics of currents under investigation in this study.

2.1.2. CELL ISOLATION

5-6 tissue pieces were incubated in nominally Ca^{2+} -free PSS (PSS containing 50 μM Ca^{2+} see 2.5.) for 20 min at 37 °C, then transferred to a 2 ml mixture of collagenase type I (2.5 mg, 289 U/mg, Sigma) and collagenase type XI (2.5 mg, 1450 U/mg, Sigma) in nominally Ca^{2+} -free PSS for 50-60 min at 37 °C. After the enzymatic digestion, tissue pieces were transferred to 4 ml of fresh nominally Ca^{2+} -free PSS, allowed to cool slowly to 4 °C and then dispersed by gentle trituration (sucking up and down through a wide-bore pipette). Cells were stored at 4 °C for up to 6 hours prior to use. For each experiment, 0.2 - 0.5 ml of cell suspension was placed on a glass coverslip fixed to perfusion chamber and cells were given 5-10 min to settle before perfusion was begun.

2.2. WHOLE CELL PATCH-CLAMP METHOD

2.2.1. THEORY

The principle of patch clamping is to maintain a potential difference across the cell membrane that is set by a user-defined command voltage. In voltage-clamp, voltage is controlled and the resultant flow of ions producing a current is measured. In current clamp by contrast, a constant controlled flow of current is put through the cell membrane and changes in membrane potential that result are measured.

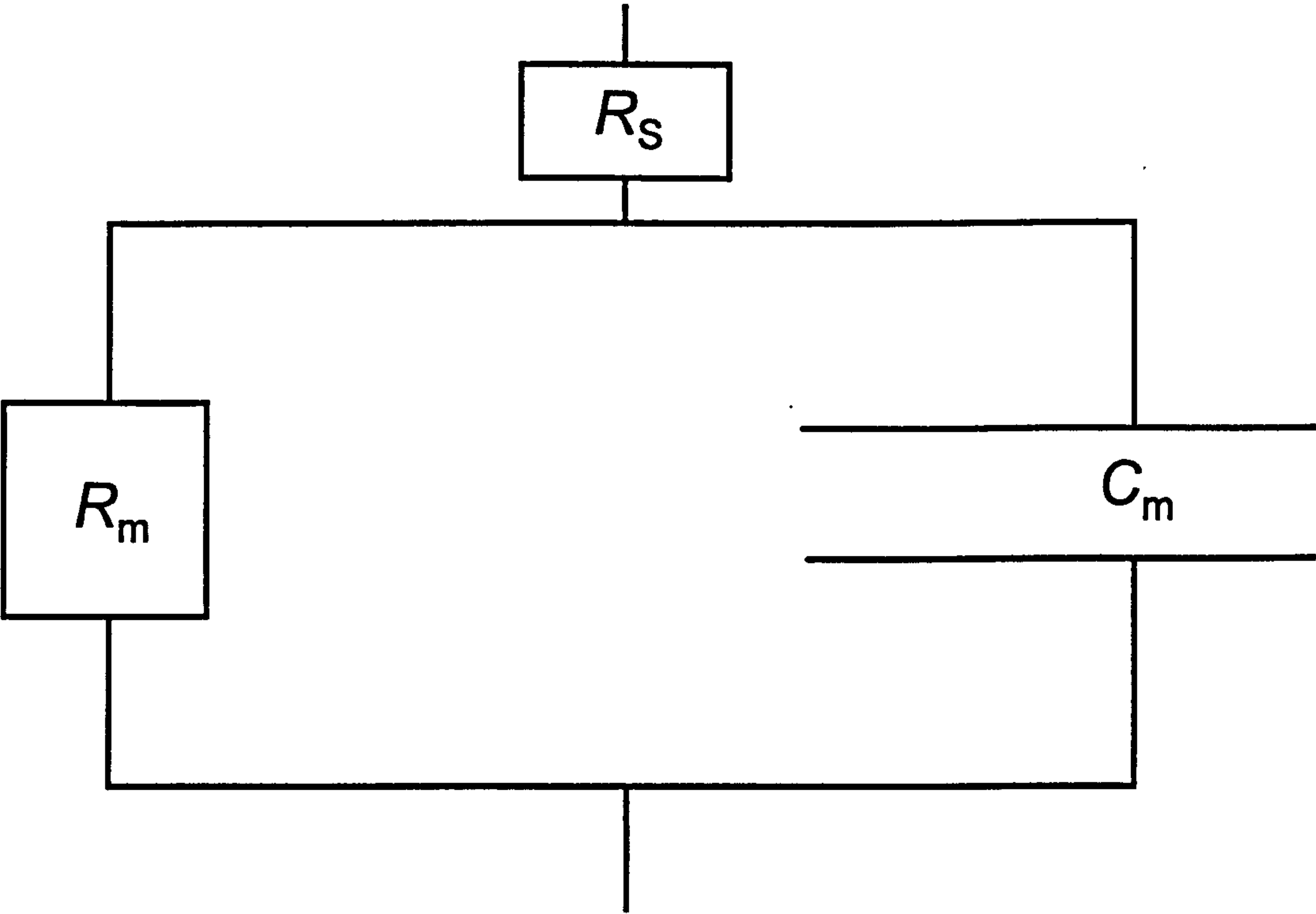
From an electrical point of view, the cell membrane may be considered as a variable resistor (R_m) and a capacitor (C_m) in parallel with each other and in series with the resistance of the pipette (series resistance or R_s) (Fig 2.1.). Total membrane current (I_m) is equal to current through the resistor (I_i) plus the capacity current (I_c). I_c however, is transient, only existing when the capacitor is charging or discharging. The amplitude of I_c changes with respect to the change in voltage with time:

$$I_c = C_m \cdot dV/dt. \quad (\text{Equ. 2.1.})$$

The patch clamp method used in this study was first described by Hamill *et al.*, (1981). Previous methods used two high resistance intracellular electrodes, one to measure the potential difference across the cell membrane and the other to inject current and maintain the cell voltage at the command level. In whole cell patch clamp a single low resistance pipette electrode is used to perform both functions. This, in theory, allows the induced potential difference across the pipette electrode to be maintained while simultaneously measuring the current. Voltage “clamping” is performed by an operational amplifier in the headstage. This compares two input voltages, V_c the user-defined command voltage and V_p the measured pipette voltage, and outputs a single voltage V_o which is proportional to

the difference between V_C and V_P . Input V_P and output V_O are connected via a negative feedback resistor (R_f) where the signal fed back to V_P through the resistor is proportional to V_O . This feedback drives V_P towards V_C , thus controlling the pipette voltage. (Fig 2.2.).

FIG 2.1. CELL MEMBRANE AS AN ELECTRICAL CIRCUIT

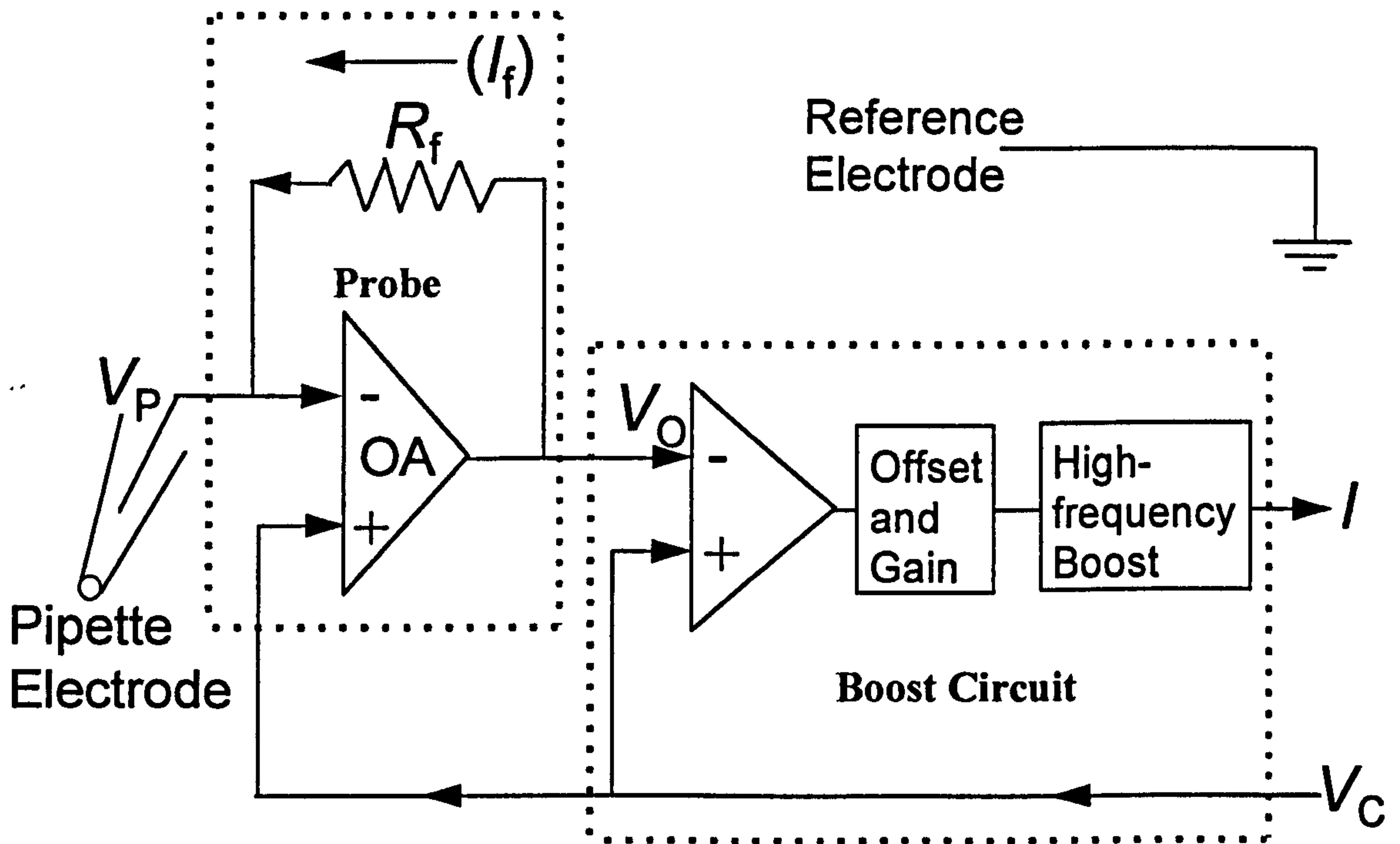


R_s = series (pipette) resistance

R_m = membrane (input) resistance

C_m = membrane capacitance

FIG. 2.2. AMPLIFIER RESISTIVE HEADSTAGE CIRCUITS



Because of the high feedback resistance of the amplifier ($500 \text{ M}\Omega$), any current flowing through the cell membrane and hence the pipette input, is equal and opposite to the current flowing through the feedback loop (I_f). The current across the membrane can therefore be measured as the voltage drop across R_f . The time it takes for V_O to settle to a final value is determined by the size of the feedback resistance (R_f) and its associated capacitance (C_{Rf}) where $\tau = R_f C_{Rf}$. Clamping of the pipette potential is accelerated and accuracy of current measurement is improved with the involvement of additional amplifiers in a boost circuit, wherein the relatively low frequency cycling of R_f is increased and junctional potentials between the pipette electrode and surrounding solutions are cancelled (see below).

Despite the boost circuit however, clamping at the cell membrane is still relatively slow because of the time it takes to charge the cell capacitor, wherein $\tau = R_p C_m$. Under voltage-

clamp therefore, membrane potential remains constant at a user-specific value (V_C) assuming that $V_P=V_C$. This is only true if current causes a negligible voltage drop across the pipette resistance. ✱

2.2.2. PIPETTE AND CELL CAPACITANCE

There are two main types of capacitance, the fast capacitance and the slower cell membrane capacitance. The fast capacitance is composed of 1) capacitance across R_f , as described above, 2) other sources within the amplifier, 3) across the holder and pipette to adjacent grounded surfaces and 4) across the pipette wall to the bath solution. The fourth type of fast capacitance may be reduced by increasing the thickness and hardness of pipette glass and by coating the tip of the pipette with Bee's wax or other hydrophobic substance. Capacitances generated within the amplifier and across R_f however, have to be compensated electronically and there is a circuit within the amplifier for this purpose. This is performed routinely for each new pipette immediately after Giga seal formation.

Cell capacitance, as described above, results in slow voltage clamp and may also obscure fast activating currents. There is also a parallel circuit for slow or cell capacitance compensation. This was not routinely used in this study because cell capacitance was occasionally found to be too large for the compensation circuit and because there is the chance that small changes in series resistance during the experiment (which slow the decay of the capacitance artefact) will potentially result in overcompensation. Cell capacitance in human myometrial cells was calculated from the area under a capacitive current induced by a -10 mV step from a holding potential of -50 mV where:

$$C_m \text{ (pF)} = \text{area (pA.ms)}/\text{step size (mV)} \quad (\text{Equ. 2.2.})$$

In 382 cells, C_m was 98.7 ± 3.4 pF.

2.2.3. SERIES RESISTANCE

Series resistance (which is equivalent to the pipette resistance in series with the membrane potential in the patched cell (Fig 2.1.), was calculated in human myometrial cells by measuring the decay rate (τ) of the capacitative current induced by a -10 mV step from a holding potential of -50 mV:

In 382 cells, τ was 1.06 ± 0.04 ms and, using the equation:

$$R_s(\text{M}\Omega) = \tau(\mu\text{s})/C_m(\text{pF}) \quad (\text{Equ. 2.3.}),$$

R_s was 10.9 ± 0.3 M Ω .

The series resistance is a source of error in membrane potential control. This is because, as has been described above, it is the pipette potential not the membrane potential which is clamped at the command voltage (V_C). If R_s , which is in series with V_P , is high there will be a large voltage drop across V_P . According to Ohm's Law, this voltage drop will be proportional to both R_s and membrane current (I), where:

$$V(\text{mV}) = R_s(\text{M}\Omega) \cdot I(\text{pA}). \quad (\text{Equ. 2.4.})$$

In pulmonary artery myocytes, the voltage discrepancy between V_C and V_P as measured with a second microelectrode was shown to be virtually identical to the predicted value (Smirnov & Aaronson, 1994). In addition, since R_s is in series with C_m , and the time constant of C_m decay, τ , is equal to $R_s C_m$, a large R_s would result in slower clamping of voltage. There is a circuit for series resistance compensation in the amplifier and, in early experiments, compensation was attempted. Unfortunately this did not prove useful in this study because small changes in series resistance (increase or decrease) during experiments

resulted in severe “ringing” capacity current oscillations. However, in this study, peak amplitudes of myometrial Ca^{2+} channel currents were never greater than 1 nA and amplitudes of K^{+} channel currents at the most depolarised membrane potential (usually +60 mV) were on average no greater than 1 nA. This means that the inaccuracy in steady-state voltage clamp (Equ. 2.4.) was on average no greater than 10 mV. Attempts were made to minimise R_s principally by using pipettes with as low a resistance as possible while still achieving a good Giga-seal (see below).

2.2.4. MEMBRANE RESISTANCE

Membrane or input resistance, which is equivalent to the resistance of the cell membrane to the passage of ions, was estimated in 18 HMSMCs, using normal physiological saline as bath solution and a high K^{+} pipette solution. In the whole cell configuration, the mean of current measured at -90, -80 and -70 mV (potentials at which no active current would be expected) in each cell was calculated. Using the equation:

$$R_m = (V_m - E_r) / I \quad \text{Equ 2.5.}$$

where R_m is the membrane resistance, V_m is the membrane potential, E_r is the reversal potential (-83 mV) and I is current, R_m was calculated in 18 cells as $0.96 \pm 0.19 \text{ G}\Omega$.

2.2.5. JUNCTION POTENTIALS

The potential recorded by a microelectrode in the bath solution (when compared to the grounded reference electrode) should ideally be zero. There is however an offset potential that arises from potential differences at the junction between the silver electrode and the pipette filling solution and between the pipette filling solution and the bath solution (junction potentials). The potential between the silver electrode and pipette filling solution is minimised by coating the electrode with silver chloride making it a non-polarisable,

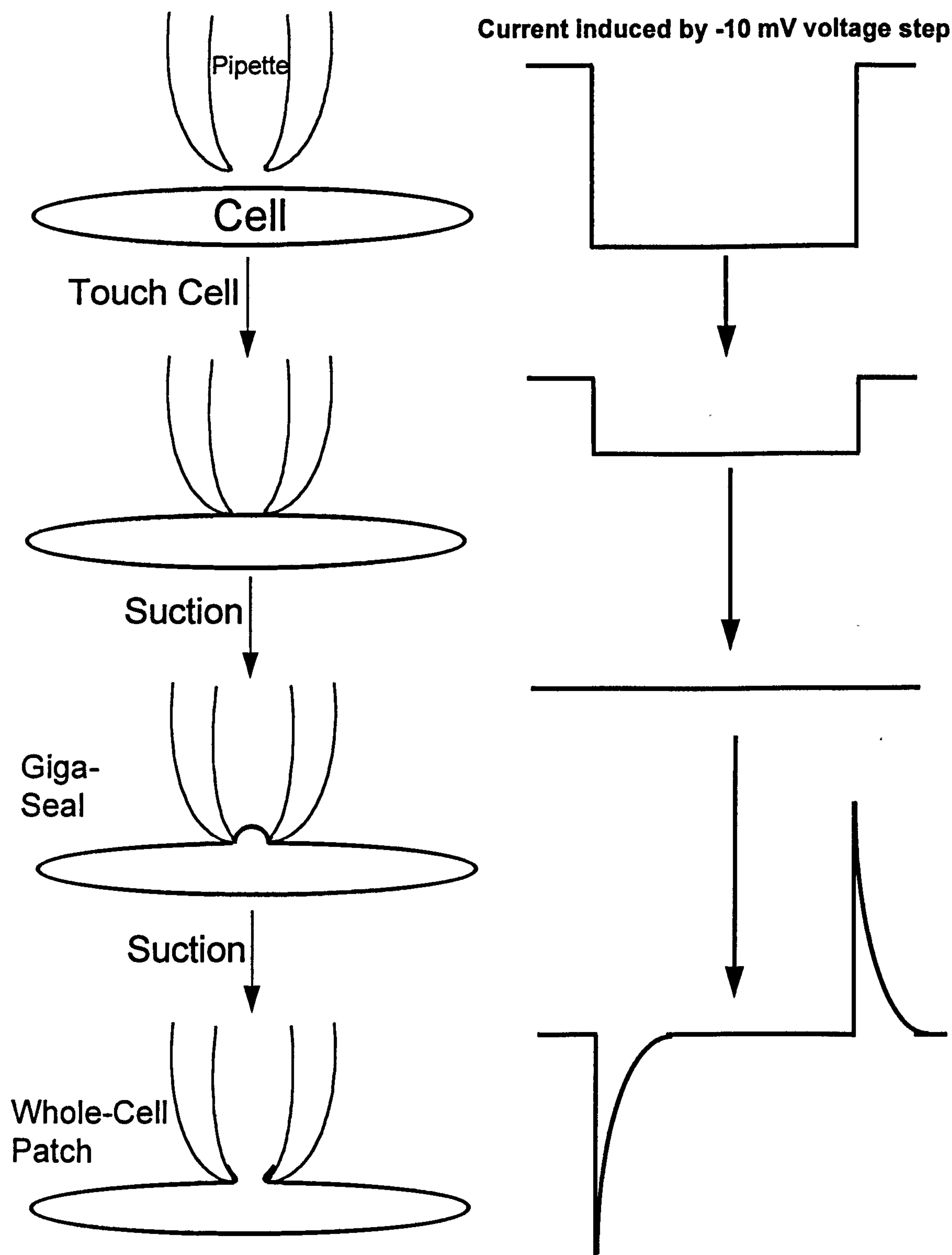
reversible Ag/AgCl electrode. The potential between pipette filling solution and bath solution is influenced by the properties of the glass pipette and the size of the tip opening, causing restriction of ionic movement in and out of the pipette, and can therefore be minimised by using wide, low resistance pipettes. The remaining offset potential is compensated for by a potentiometer in the patch-clamp amplifier which adds \pm mV to the command potential while the pipette is in the bath solution. The reference electrode is also Ag/AgCl in composition.

2.2.6. GIGA-SEAL AND WHOLE-CELL PATCH FORMATION

The procedure for producing whole-cell patches is summarised in Fig 2.3. A very high resistance seal of the pipette tip (usually several Giga Ohms ($G\Omega$)) onto the cell surface is achieved by prior heat-polishing of the pipette tip by bringing it close to near-red hot platinum wire for a few seconds and attempting to produce dispersed cells free of extracellular connective tissue proteins. The resistance is monitored by applying a continuous rapid 10 mV step and observing the amplitude of the current step induced. With the pipette in the bath, current amplitudes range from 1-3 nA, giving pipette resistances of 3-10 $M\Omega$. The pipette tip may be kept clean by applying slight positive pressure while lowering the pipette towards the cell. Giga-seal is usually achieved by slight suction after initial contact of the pipette with the cell. When suction is applied, the current amplitude is reduced to between 0.5 and 2 pA, giving seal resistances of between 5 and 20 $G\Omega$.

Following Giga-seal formation, slightly stronger suction is applied to the pipette in order to rupture the patch of membrane contained within the tip of the pipette and gain electrical access to the inside of the cell. Successful electrical access to the cell interior is determined by the appearance of large cell capacititive current transients. The advantages of high seal resistances are two-fold. Firstly, the background noise is reduced allowing small currents

FIG. 2.3. PROCEDURE FOR TIGHT-SEAL WHOLE-CELL PATCH FORMATION



(~1 pA) to be recorded, and secondly, the inner surface of the cell membrane becomes electrically isolated, allowing it to be voltage-clamped without the need of a second microelectrode (Sigworth & Neher, 1980).

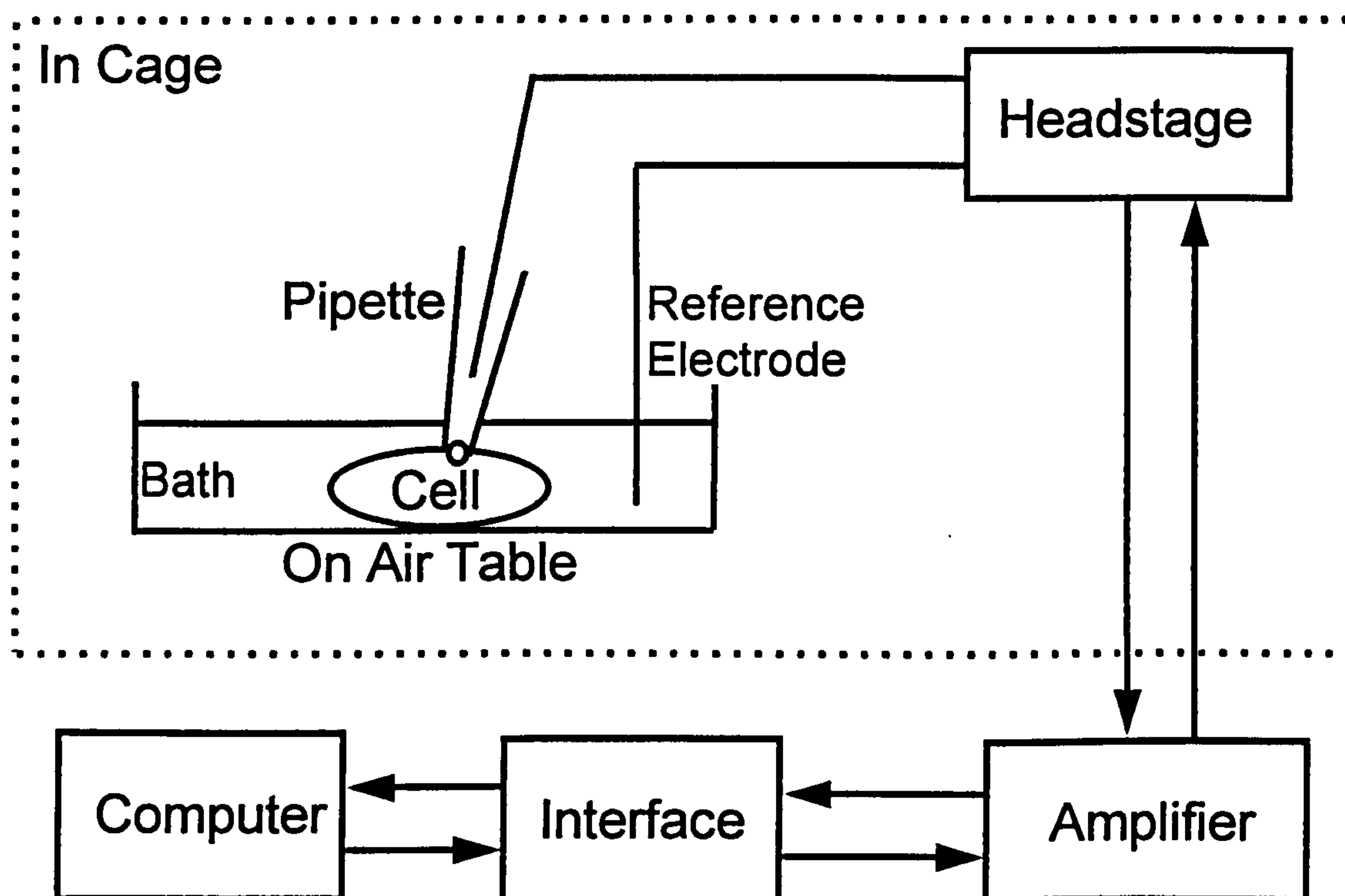
2.2.7. PIPETTE PULLING

Electrode pipettes were pulled from 225 pcs. borosilicate glass capillaries (1.5 mm O.D. x 0.86 mm I.D., Clark Electromedical Instruments) using a two-step heat-adjusted pipette puller (Narishige, Japan) and polished with a hot platinum wire.

2.2.8. ELECTROPHYSIOLOGY SET-UP

Pipettes were fixed and supported in an HL-U pipette holder connected to the headstage. The holder was connected to a valve-operated syringe for the application of suction. Vibration was minimised by mounting both microscope (Nikon Diaphot 300) and micro-manipulator (MP-285, Sutter Instrument Company) on a gas-inflated table (Melles Griot). 50 Hz electrical noise was minimised by surrounding sensitive equipment in a Faraday cage and grounding to the amplifier signal ground socket. Cells were continuously perfused with bath solution with an approximate flow rate of 1 ml per minute (input by gravity feed and output by vacuum pump). Exchange of solutions was rapid: complete exchange, as assessed by monitoring the change in pipette resistance when saline was replaced with distilled water, was in 10-20 seconds. The organisation of the electrophysiology set-up is summarised in Fig 2.4. (below).

FIG. 2.4. ELECTROPHYSIOLOGY SET-UP



2.3. SOLUTIONS AND CHEMICALS

2.3.1. BATH SOLUTIONS FOR Ca^{2+} AND K^+ CHANNEL CURRENTS

The standard constituents of bath solutions are listed in Table 2.1. Ca^{2+} channel currents were recorded using either 1.5 mM Ca^{2+} (I_{Ca}) or 10 mM Ba^{2+} (I_{Ba}) as charge carrier. I_{Ca} and I_{Ba} were recorded in the presence of 4 mM TEA and 1 mM CsCl to inhibit residual K^+ current.

TABLE 2.1. BATH SOLUTIONS FOR RECORDING Ca^{2+} AND K^{+} CHANNEL CURRENTS (mM)

Bath Solution	$\text{Ca}^{2+}/\text{Cs}^{+}/\text{TEA}$ PSS for I_{Ca}	$\text{Ba}^{2+}/\text{Cs}^{+}/\text{TEA}$ PSS for I_{Ba}	Normal Ca^{2+} PSS for I_{K}	" Ca^{2+} -free" PSS for I_{K}
NaCl	120	120	130	130
KCl	-	-	5	5
MgCl_2	1.2	1.2	1.2	1.2
$\text{CaCl}_2/\text{BaCl}_2$	1.5 (Ca^{2+})	10 (Ba^{2+})	1.5 (Ca^{2+})	0.015 (Ca^{2+})
CsCl	1	1	-	-
TEA-Cl	4	4	-	-
HEPES	10	10	10	10
glucose	10	10	10	10

K^{+} channel currents were recorded using normal PSS or Ca^{2+} -free PSS. The same two solutions were used for storage and enzymatic dispersal of myometrial tissue, respectively. Any additions or alterations to bath solutions are described in the relevant chapters. All bath solutions were corrected to pH 7.4 with NaOH unless otherwise stated in individual chapters.

2.3.2. PIPPETTE SOLUTIONS FOR Ca^{2+} AND K^{+} CHANNEL CURRENTS

The standard constituents of the pipette solutions are listed in Table 2.2. For I_{Ca} or I_{Ba} , NaCl was replaced by CsCl to inhibit outward K^{+} currents and isolated inward Ca^{2+}

currents. ATP was present in pipette solutions to prevent run-down of Ca^{2+} channel current by acting as a substrate for channel phosphorylation by kinases (Lorenz & Paul, 1997). Mg^{2+} was also required as a co-factor for phosphorylation. The combination of 1.5 mM Ca^{2+} and 10 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) in the pipette solution resulted in a free intracellular Ca^{2+} concentration of 8 nM (Fabiato & Fabiato, 1979). Any additions or modifications to pipette solutions are described in the relevant chapters and, unless otherwise stated, pipette solutions were buffered to pH 7.2 with NaOH. All pipette solutions were filtered (0.2 μM pore size) immediately prior to use to avoid clogging of the pipette tip.

TABLE 2.2. PIPETTE SOLUTIONS FOR RECORDING Ca^{2+} AND K^{+} CHANNEL CURRENTS (mM)

Pipette solution	For $I_{\text{Ca}}/I_{\text{Ba}}$	For I_{K}
CsCl	135	-
KCl	-	110
MgCl_2	2.5	2.5
MgATP	5	1
CaCl_2	1.5	-
HEPES	10	10
EGTA	10	10

2.3.3. DRUGS AND CHEMICALS

Paxilline, tetraethylammonium (TEA), 4-aminopyridine (4-AP), oxytocin, indomethacin and prostaglandins $F_{2\alpha}$ and E_2 were all purchased from Sigma UK. Clofilium tosylate was obtained from R.B.I. Research Biochemicals (Natick, MA, USA). Nimesulide (N-(4-nitro-2-phenoxyphenyl)-methane-sulfonilamide) was a gift from Dr Robert Sawdy (Queen Charlottes Hospital, London). Mibefradil {(1*S*,2*S*)-2-[2[3-(2-benzimidazolylpropyl)methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate dihydrochloride} was a gift from Dr Mike Shattock (Rayne Institute, London). PSS constituents were obtained from BDH (Poole, UK). Nimesulide, indomethacin, paxilline, clofilium, 4-AP and the prostaglandins were prepared from stock solutions in dimethylsulphoxide (DMSO). All other drugs were dissolved in distilled water.

2.4. DATA AQUISITION AND ANALYSIS

Currents were recorded, via CV 203BU headstage, Axopatch 200B amplifier and Digidata 1200-series interface (Axon Instruments Inc., CA, USA), onto computer (Elonex MTX-6233/II), using pCLAMP version 6.03 (CLAMPEX component) software (Axon Instruments Inc.). High frequency noise was reduced during recording with the amplifier lowpass bessel filter set to 1 KHz for Ca^{2+} currents or 2 or 5 KHz for K^+ currents.

2.4.1. TRACE ANALYSIS AND LEAK SUBTRACTION

Raw data was analysed using the CLAMPFIT component of pCLAMP 6 software. Data (current measurements or selected traces) was then exported, first into Microsoft Excel for statistical analysis, and then into SigmaPlot (version 4.1.) for non-linear regression curve fitting and/or graph plotting (see relevant chapters for curve equations).

Leak subtraction was performed in CLAMPFIT, rather than during recording. Currents were only recorded from cells where the passive “leak” (defined as current flowing at membrane potentials not expected to open voltage-sensitive ion channels under the conditions used) was below acceptable levels. This was usually less than 10 % of total current. CLAMPFIT contained an algorithm which, using the measured current at the holding potential (either -50 or -80 mV) and the estimated input resistance (assumed to be a linear relationship between leak current amplitude and membrane potential), calculated the component of leak current scaled to all points in the voltage protocol and subtracted this from the current trace. The advantage of this technique was that changes in leak from one trace to the next could be corrected individually.

2.4.2. STATISTICS

Statistical comparisons between two sample means were made using Student’s two-tailed un-paired t-test when comparing groups of data derived from different cells, or two-tailed paired t test when comparing groups of data obtained from the same cells. Comparisons between three or more sample means were made using two-tailed analysis of variance (ANOVA). Differences were considered significant when $P < 0.05$.

All data are expressed as the mean and standard error of the mean (S.E.M.).

Chapter 3

Calcium Channel Currents in HMSMCs of Pregnant Women

3.1. METHODS AND RESULTS

3.1.1. EFFECT OF Ba^{2+} AND Cd^{2+} ON INWARD CURRENT IN HMSMCS

All experiments in this chapter were performed with freshly dispersed HMSMCs from pregnant women at term who were not in active labour. Experiments in this chapter were performed using the Cs^+ /TEA-containing PSS bath solution, with either 1.5 mM Ca^{2+} for I_{Ca} , or 10 mM Ba^{2+} for I_{Ba} (Table 2.1.), and the Cs^+ -containing pipette solution, designed to inhibit K^+ currents (Table 2.2.). The inward current recorded from a holding potential (V_{H}) of -50 mV via a depolarising step to 0 mV with 1.5 mM Ca^{2+} in the bath solution (I_{Ca}) was enhanced by replacement of Ca^{2+} with 10 mM Ba^{2+} (I_{Ba}). Peak current amplitude (measured at 20-30 ms for I_{Ca} or 30-50 ms for I_{Ba}) in 6 cells was increased 5.9 ± 1.2 times. In addition, decay of I_{Ba} was significantly slower than that of I_{Ca} . In the 6 cells, current at the end of 300 ms depolarisations to 0 mV had decayed by 99 ± 11 % in Ca^{2+} and by 62.7 ± 6.9 % in Ba^{2+} ($P < 0.05$).

Ca^{2+} channel current was also inhibited in a dose dependent fashion by CdCl_2 , both I_{Ca} and I_{Ba} being inhibited by micromolar concentrations. The dose-dependence of the effect on I_{Ca} was determined at +10 mV from V_{H} -50 mV (Fig 3.1.). The data in Fig 3.1.B is fitted to a 4-parameter logistic function (Langmuir equation):

$$y = (100-a)/(1 + ((\text{EC}_{50}/x)^b)), \quad \text{equ. 3.1.,}$$

where y is the percent inhibition at each concentration, x is the micromolar concentration, EC_{50} is the half-maximal inhibitory concentration, a is the residual component of inhibition and b is the Hill coefficient.

The EC₅₀ of the fitted curve was 1.47 μ M, a was 4.64 and the Hill coefficient was 1.4. Complete inhibition of all inward current by CdCl₂ (either I_{Ca} or I_{Ba} , see Fig 3.1.C) was achieved with 50 μ M or higher. Block with CdCl₂ was completely and readily reversible (not shown). Because of the greater current amplitude and consequent ease of analysis, most of the subsequent Ca²⁺ channel current experiments were performed with Ba²⁺ as the charge carrier (I_{Ba}).

3.1.2. ELECTROPHYSIOLOGICAL CHARACTERISTICS OF I_{Ba}

The electrophysiological characteristics of Ca²⁺ channel currents (using I_{Ba}) were determined by investigating the voltage-dependence of activation (with current voltage (I - V)-relationships) and inactivation (availabilities). Figure 3.2. shows the I - V of I_{Ba} , from V_H -80mV and -50 mV (Figure 3.2.A). The current activated at a relatively negative potential (-60 mV) from V_H -80 mV, and demonstrated an initial rapid component of decay which was not apparent when the holding potential was -50 mV, in which case the apparent threshold of activation was around -40 mV. When the currents elicited from V_H -50 mV were subtracted from the currents evoked from V_H -80 mV, a rapidly inactivating current with negative threshold of activation was observed. This rapidly-inactivating component of current reached a maximal amplitude at -20 mV, while the slowly-inactivating current evoked from V_H -50 mV reached its peak between +10 and +20 mV (Figure 3.2.B). The differences in kinetics and voltage-dependence of current activation are suggestive of T-type and L-type currents respectively. The terms T-component and L-component are hereafter used to describe the two current components. The difference in decay rate of L- and T-components of I_{Ba} was confirmed by fitting current traces to a single component exponential function:

$$y = A \cdot \exp(-t \cdot \tau) + B \quad \text{equ 3.2.,}$$

where A is the current amplitude at any given time point, t is the time point, τ is the rate constant and B is the non-decaying component. τ for the L-component at +10 mV from V_H -50 mV (211 ± 29 ms, $n = 12$) was significantly greater than for the T-component at -20 mV from V_H -80 mV (37 ± 4 ms, $n = 7$) ($P < 0.001$).

Figure 3.3A shows the availability of I_{Ba} at +20 mV and -20 mV test potentials, observed using a range of 5 s conditioning potentials from -100 mV and +20 mV. The current at +20 mV, measured at its peak (shown in Fig 3.3.B, open squares), demonstrated a biphasic inactivation curve which was fitted with a double component sigmoidal equation (also known as a Boltzmann function):

$$y = A/[1 + (\exp((V_m - V_{0.5T})/k_T))] + B/[1 + \exp((V_m - V_{0.5L})/k_L)] + C \quad \text{Eqn 3.3.,}$$

where y is the normalized current at any potential, V_m is the membrane potential, $V_{0.5T}$ and $V_{0.5L}$ are the half-inactivation potentials, k_T and k_L are the slope factors of the low threshold (A, T-component) and high threshold (B, L-component) currents, respectively; and C is the component of current not inactivated. $V_{0.5T}$ was -68.4 ± 4 mV and $V_{0.5L}$ was -19.4 ± 2 mV. k_T was 6.72 ± 1.3 mV and k_L was 6.73 ± 0.85 mV. The A component was 36.8 ± 6.8 % and the B component 56.5 ± 7.3 % of the total current in 9 cells.

The T-component should be most obvious at the peak of the current, and also at more negative test potentials, where it is prominent and the L-component is only slightly activated. Conversely, the L component should predominate at more positive test potentials, and at the end of the pulse (300 ms), since the T-component will have almost completely inactivated. The availability of the end-of-pulse current at a test potential of +20 mV (L-component, filled squares) was therefore compared with that of the peak current at a test potential of -20 mV (T-component, filled circles), elicited following 5 sec conditioning potentials, using a single component Boltzmann function:

$$y = A/[1+\exp((V_m-V_{0.5})/k)]+A \quad \text{Eqn 3.4.}$$

The end of pulse currents at the +20 mV test pulse had a high voltage-range of inactivation ($V_{0.5} = -16.4 \pm 7.6$ mV, $k = 7.65 \pm 1.0$ mV, $A = 9.22 \pm 3.84$ %, $n=8$; Figure 3.3B, solid squares). By contrast, the peak currents at the -20 mV test pulse had a low threshold of inactivation ($V_{0.5} = -65.3 \pm 1.8$ mV, $k = 4.94 \pm 2.4$ mV, $A = 5.46 \pm 0.54$ %, $n=6$; Figure 3.3B, solid circles). These values were not significantly different than the $V_{0.5T}$ and $V_{0.5L}$ derived from the double-component Boltzmann fitting of the overall current peak at +20 mV.

Taken together, the results of Figures 3.2. and 3.3. indicate that HMSMCs exhibit two distinct components of Ca^{2+} channel current; a T-type component with negative ranges of activation and inactivation and a rapid decay, and an L-type component with more positive ranges of activation and inactivation, and slower inactivation kinetics.

3.1.3. PHARMACOLOGY OF L- AND T-COMPONENTS OF I_{Ba}

Mibefradil, a reportedly T-type selective Ca^{2+} channel antagonist inhibited both T- and L-components of I_{Ba} in HMSMCs. This inhibition was apparently voltage dependent. 300 ms duration currents were recorded in 4 cells over a range of membrane potentials from $V_H - 80$ mV. Figure 3.4A shows an example of the effect of 1 μM mibefradil on currents at potentials from -50 mV through to +40 mV at 10 mV increments. At -50, -40, -30 and -20 mV the transient low threshold T-component which predominated at these potentials was nearly completely blocked, whereas the high threshold slowly decaying L-component, which began to emerge at -20 and -10 mV was poorly affected at those potentials. At potentials above 0 mV however, where the L-component predominated, inhibition was enhanced as the membrane potential became more positive. In addition, the decay rate of the L-component at potentials positive of 0 mV was increased by 1 μM mibefradil. For

example, from fitting currents to a single exponential decay (equ. 3.2), τ , at +20 mV, was 89 ± 4 ms in PSS vs. 54 ± 8 ms in mibefradil, $P < 0.01$, and at +30 mV, it was 86 ± 5 ms in PSS vs. 55 ± 8 ms in the presence of mibefradil, $P < 0.05$, in the 4 cells studied.

These currents were measured at the peak (30-50 ms) and end of pulse (275-300 ms) in the presence of 0 (PSS), 0.1 and 1 μ M mibefradil, normalised to the current amplitude at 0 mV in PSS and summarised in Figure 3.4B (peak) and 3.4C (end of pulse). The apparently greater inhibition of the T-component (at -30 mV, peak) than of the L-component (at +10 mV, peak) by 0.1 μ M mibefradil was statistically significant (% inhibition at -30 mV was 62.5 ± 11.5 % vs. 35.9 ± 9.7 % for +10 mV, $P < 0.05$ by paired t-test, $n = 3$). The L-component was clearly blocked by 1 μ M mibefradil more potently at the end of the pulse than at the peak, although this was only statistically significant at 0 mV ($P < 0.05$, $n = 4$). Furthermore, at the end of the pulse (Figure 3.4C), where the L-component predominates, the potential of maximum current amplitude was apparently shifted to the left by approximately 10 mV, as indicated by the enhancement of end of pulse current by 0.1 μ M mibefradil at -20 mV (current increased by 55.5 ± 21.5 %, $P < 0.05$, $n = 3$) compared to inhibition at potentials positive of -10 mV (eg. current inhibited by 79 ± 16.6 % at +20 mV, $P < 0.01$, $n = 3$).

Availability experiments in the presence of mibefradil were not performed. However, an effect on the voltage-dependence of inactivation of the L-component in 4 cells was suggested by the near complete inhibition of the L-component at +20 mV (both peak and end of pulse) remaining in the presence of 1 μ M mibefradil when the V_H was -50 mV, its partial recovery (but with faster decay than in control currents) when the holding potential was lowered to -80 mV (without changing the test potential), and then its block again when the V_H was returned to -50 mV (Fig 3.4D).

3.1.4. FAST Na⁺ CURRENT IN HMSMCS

To determine whether a fast Na⁺ current contributed to the low threshold transient T-component of I_{Ba} , current was recorded at -20 mV from V_H -80 mV in the presence of Na⁺ and then when NaCl in the bath solution was replaced by TEA-Cl. In three cells, removal of Na⁺ did not inhibit the current (not shown). Furthermore, inhibition of I_{Ba} with 1 μ M mibefradil in the same cells did not reveal a very rapidly activating and inactivating inward current. In a very few cells however (5 out of approximately 150 cells), a very rapidly activating and inactivating inward current superimposed on the L-component was observed, suggestive of a Na⁺ current. The current voltage relationship in one cell, with normal Ba²⁺- and Na⁺- containing bath solution, is shown in Figure 3.5. From V_H -50 mV, two components to the current are clearly visible. The fast component (I_{Na}) decayed within 10 ms and the slow component (L-component of I_{Ba}) was only partially decayed after 300ms. I_{Na} in this cell activated at potentials positive of -30 mV, reached a maximum amplitude at +10 mV and appeared to reverse at around +50/+60 mV. The availability of I_{Na} in the same cell was also performed. Inactivation was voltage-dependent with a $V_{0.5}$ of -31.1 mV (using equ 3.4). I_{Na} inactivation rate was estimated in 5 cells by fitting currents at +10 mV from V_H -50 mV to a single exponential function (using equ. 3.1). The decay constant (τ) of this very fast current was 2.01 ± 0.11 ms. This compares with the T-component of I_{Ba} , which is present in the vast majority of cells, of which τ was 37 ± 4 ms in 7 cells.

3.1.5. SPONTANEOUS AND OXYTOCIN-INDUCED CHANGES IN I_{Ca} AND I_{Ba} AMPLITUDE

In order to examine the effect of oxytocin on I_{Ca} or I_{Ba} and at the same time to determine the stability of current over long periods, 300 or 500 ms currents at -10 or 0 mV from V_H -60 mV were recorded at a frequency of 0.1 Hz for up to 15 min in 18 cells (9 with I_{Ca} and 9 with I_{Ba}). Current amplitude was estimated using the area enclosed by each current trace

(the integral). In the majority of cells, after an initial run-up, current amplitude was steady for prolonged periods with no evidence of run-down (Fig 3.6A), and on average there was a small degree of run-up (in Ba^{2+} $12 \pm 20\%$ at 5 min, $18 \pm 31\%$ at 10 min and $17 \pm 32\%$ at 15 min, $n = 6$, and in Ca^{2+} $12 \pm 13\%$ at 5 min and $11 \pm 11\%$ at 10 min, $n = 4$). In a small proportion of cells however (6 out of 18 cells), oscillations in current amplitude both in I_{Ca} ($n = 2$) and I_{Ba} ($n = 4$) occurred, apparently spontaneously (Fig 3.6B), sometimes after a 5-10 min period of quiescence. These oscillations varied in amplitude (between 20 and 60 % reduction of current amplitude at the trough of the oscillation), occurred approximately once every 1-2 min and continued throughout the recording period.

To test the buffering power of the 10 mM EGTA in the pipette, the effect of 5 μM (in 3 cells) and 10 μM (in 3 cells) cyclopiazonic acid (CPA, an SR Ca^{2+} ATPase inhibitor) in the bath solution on I_{Ba} was determined. In all 6 of these cells, no spontaneous oscillations in I_{Ba} were observed during a 10-20 min recording period, but CPA, applied after the control period, inhibited I_{Ba} ($27.8 \pm 7.3\%$ by 5 μM and $31.1 \pm 7.3\%$ by 10 μM , inhibition of integrated current), suggesting that even after at least 5 min perfusion in Ca^{2+} free PSS and dialysis with 10 mM EGTA, intracellular Ca^{2+} may still be released in sufficient quantity to inhibit the Ca^{2+} channel current. This inhibition was not reversible after 5-10 min washout of CPA. In a further 3 cells, I_{Ba} was recorded with 30 mM EGTA, 20 mM 1,2-bis(2-aminophenoxy)-ethane- N,N,N',N' -tetraacetic acid (BAPTA) in the pipette solution to more firmly buffer intracellular Ca^{2+} and with heparin in the pipette solution to prevent release of Ca^{2+} from internal stores. Despite these measures, spontaneous oscillations still occurred in two of three cells.

The effect of 30 nM oxytocin (OT) was examined in 15 cells (6 in I_{Ca} and 9 in I_{Ba}), in which the current was not oscillating when OT was added. In 10 cells (5 with I_{Ca} and 5 with I_{Ba}) OT produced significant inhibition of the current (example in Fig 3.7A). This inhibition had two components, a transient component (inhibition was $64.6 \pm 16.5\%$ for

I_{Ca} and 58.7 ± 13.2 % for I_{Ba}) followed by a sustained component (inhibition was 23.5 ± 3.4 % for I_{Ca} and 26.2 ± 6.5 % for I_{Ba} , see Fig 3.7. for statistics). Measurements of current density for these cells in the control period, during the transient and sustained components of the inhibition by OT and after washout, are presented in Figures 3.7B and 3.7C for I_{Ca} and I_{Ba} , respectively. In two cells, after an initial transient inhibition OT appeared to induce oscillations in I_{Ca} (1 cell) and I_{Ba} (1 cell) that were similar in appearance to the spontaneous oscillations in other cells (not shown). These oscillations persisted after OT was washed out.

FIG. 3.1. EFFECT OF CADMIUM ON I_{Ca} AND I_{Ba} IN HMSMCS

Ca^{2+} channel currents elicited by 250 ms depolarisations from V_H -50 mV to +10 mV using either 1.5 mM Ca^{2+} (I_{Ca}) or 10 mM Ba^{2+} (I_{Ba}) as charge carrier. An example of the cumulative dose-dependent inhibition of I_{Ca} by 1, 2, 5 and 10 μ M $CdCl_2$ (indicated by arrows) is shown in **A** and the mean \pm S.E.M. inhibition of integrated current (pA.ms) in 7 cells is shown in **B**. The effect of 50 μ M $CdCl_2$ (indicated by arrow) on I_{Ba} is shown in **C**. The dotted lines represent zero current.

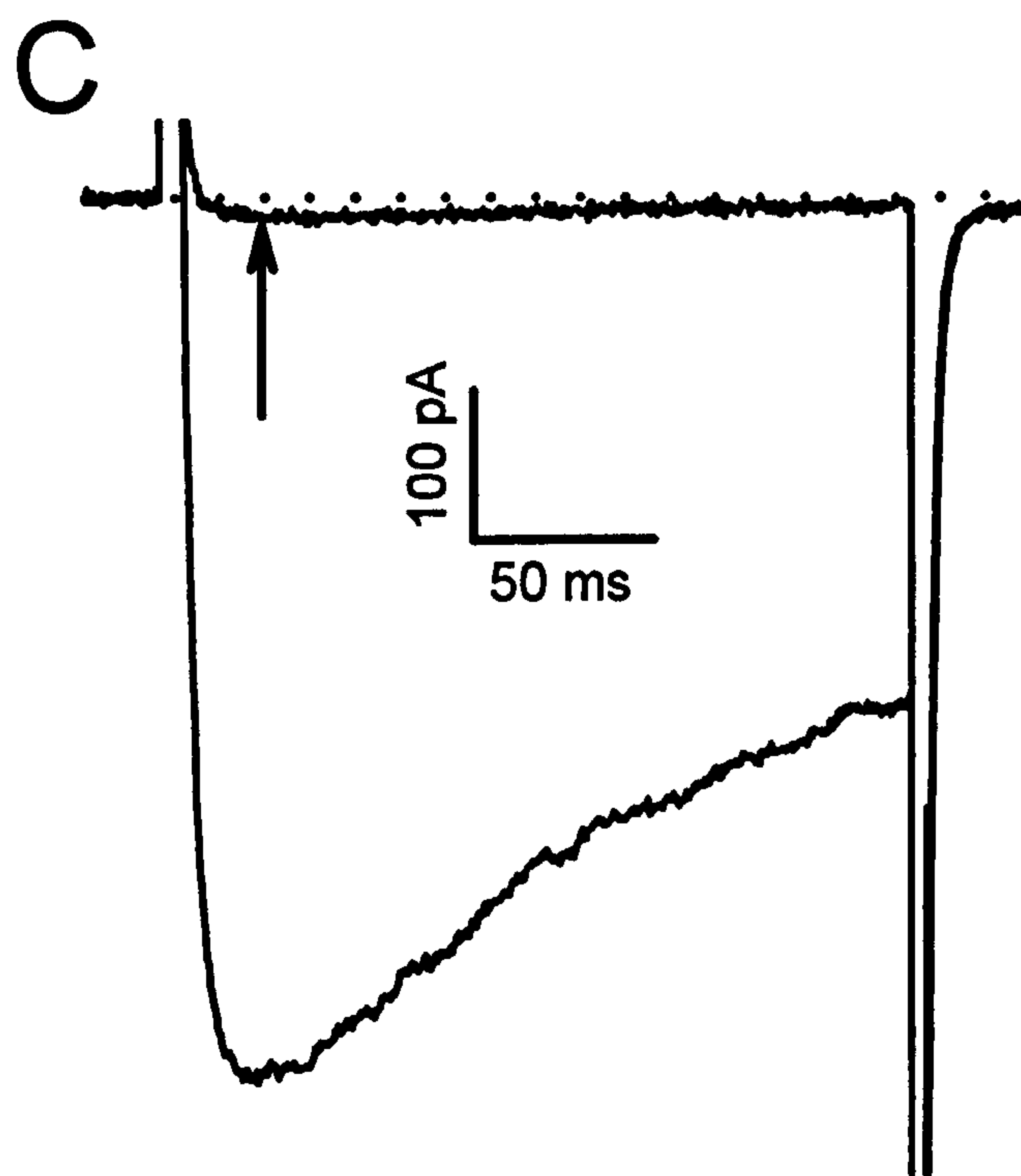
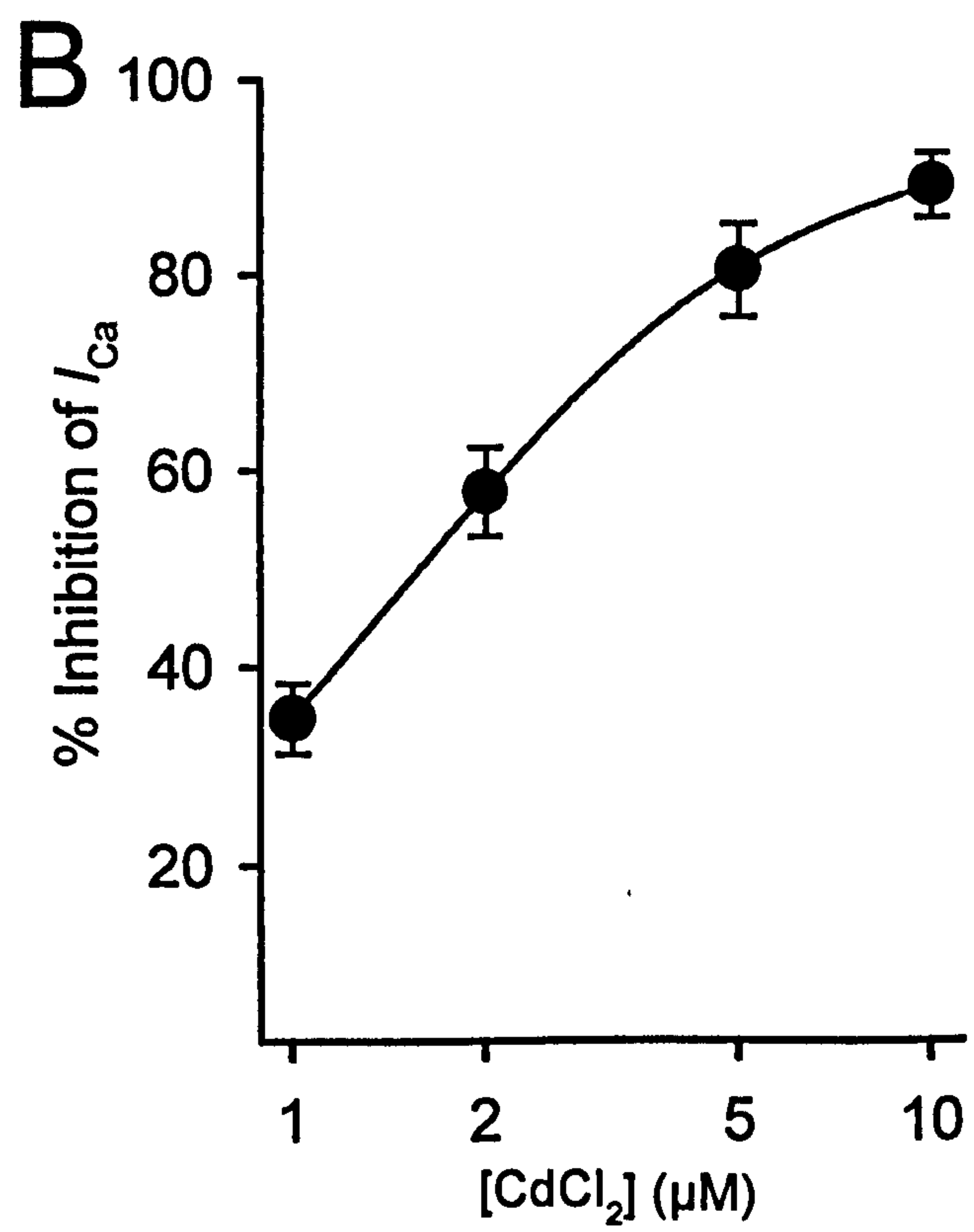
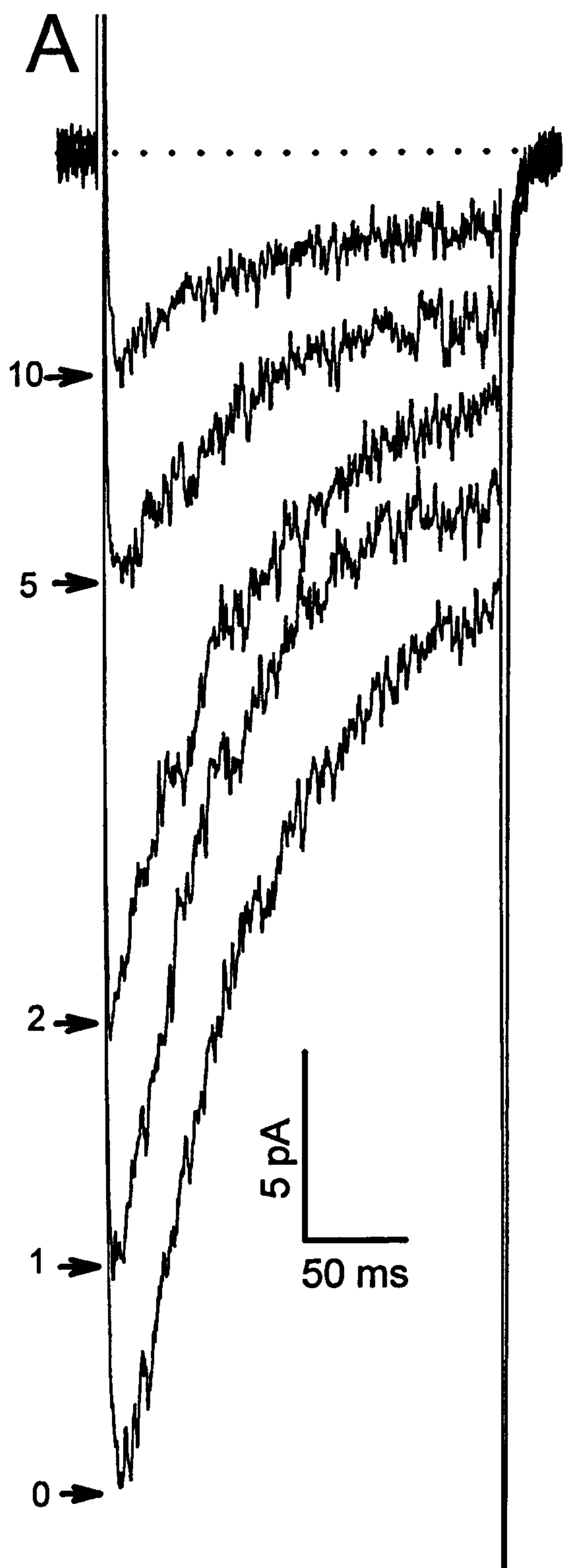


FIG 3.2. CURRENT VOLTAGE RELATIONSHIP OF I_{Ba} IN HMSMCS.

A: Currents elicited by 300 ms depolarizations to between -60 mV and +60 mV in a representative cell, from V_H of either -80 mV or -50 mV, and the difference (subtracting currents from V_H -50 mV from currents from V_H -80 mV). **B:** Peak amplitude of currents held at -80 mV (\blacktriangledown), -50 mV (\bullet) and the difference (\blacksquare), normalised to the +10 mV pulse from the -50 mV holding (mean \pm S.E.M. in the same 6 cells). The dotted lines represent zero current.

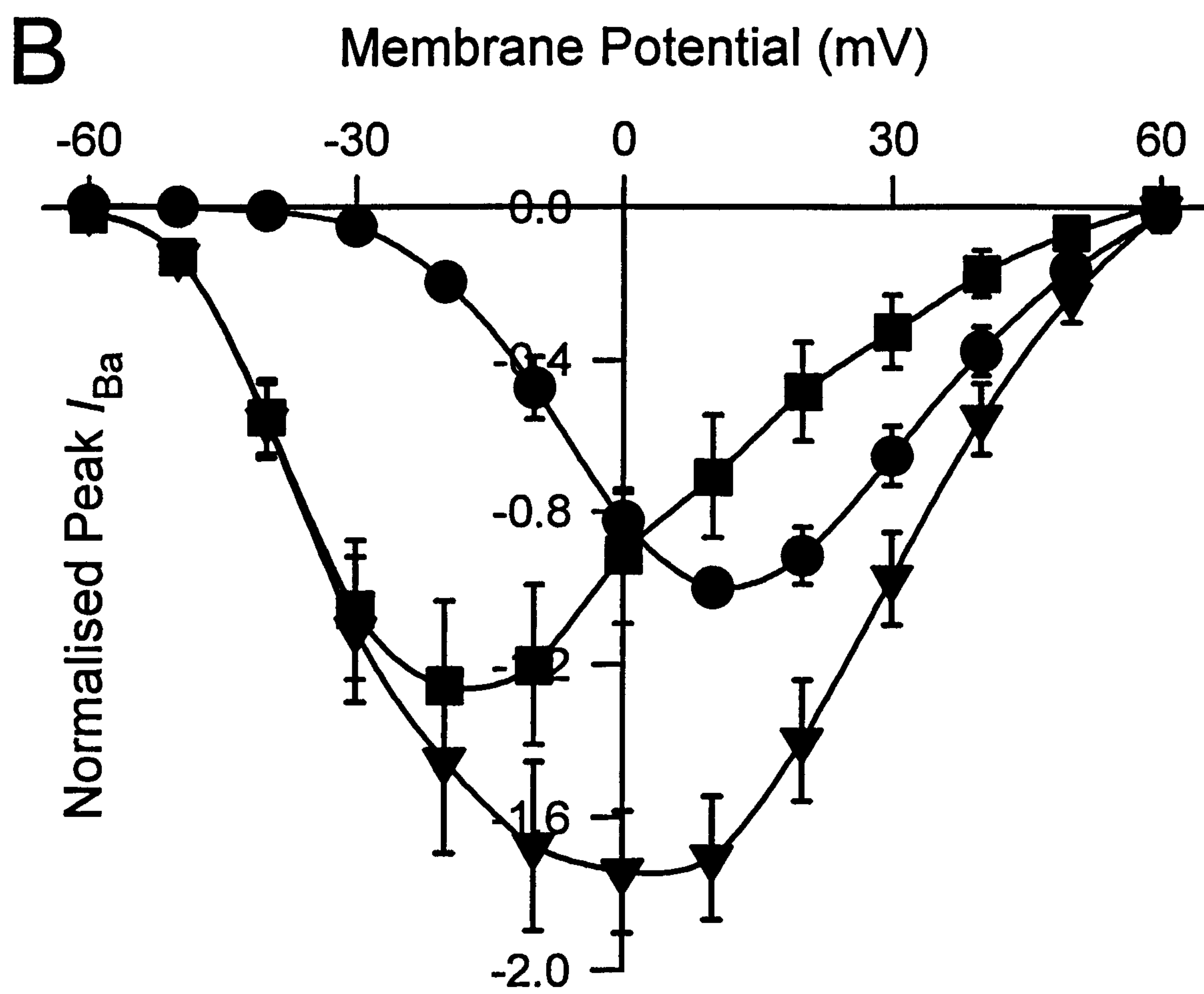
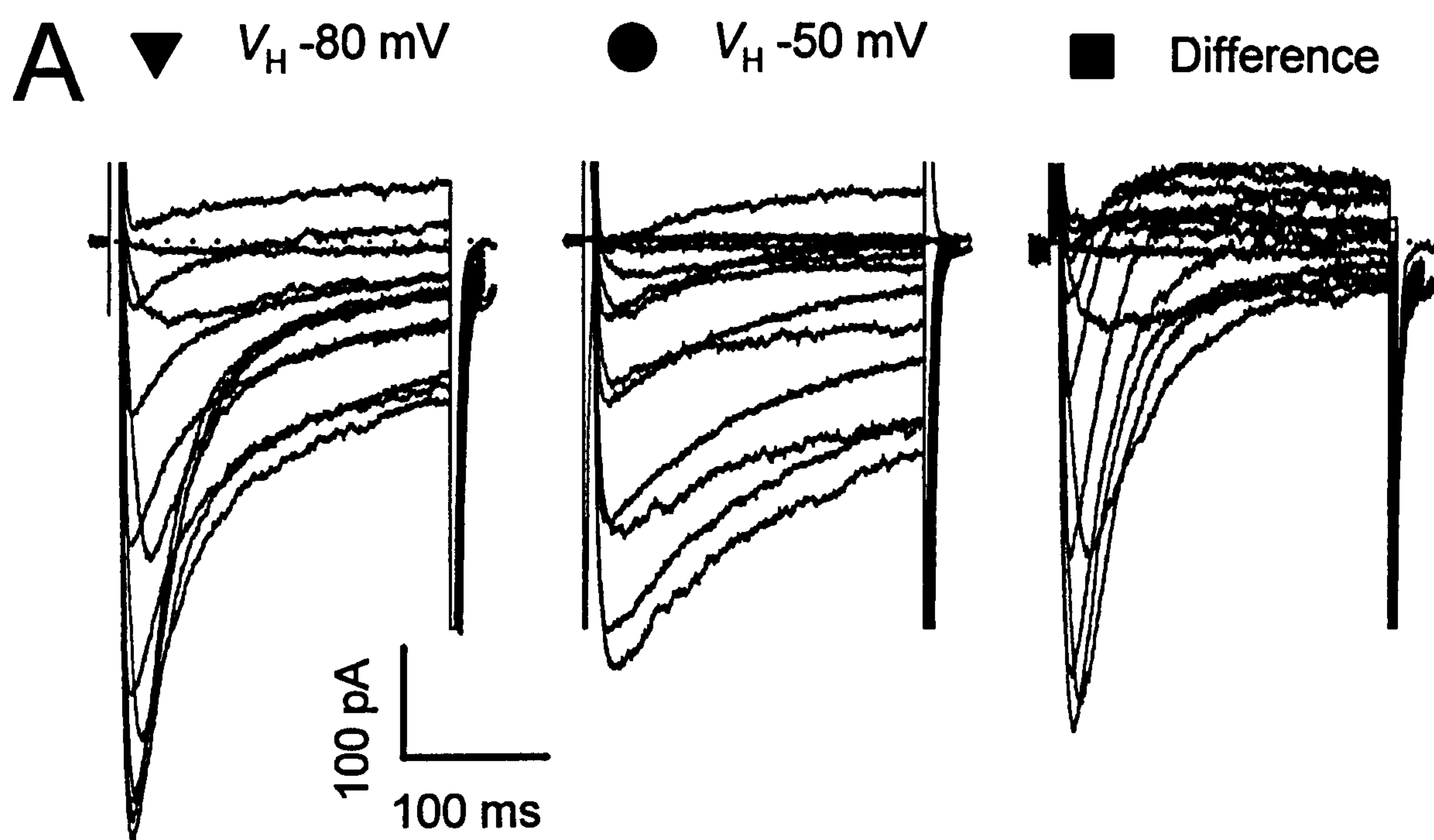


FIG. 3.3. AVAILABILITY OF Ca^{2+} CURRENTS IN HMSMCS

Availability of I_{Ba} in HMSMCs. A: Currents in a representative cell elicited by 300 ms depolarizations to +20 mV or -20 mV following 5 s preconditioning pulses to between -100 mV and +20 mV. B: Points are the mean \pm S.E.M. of peak current at -20 mV (●, n=6), peak current at +20 mV (□, n=9) and currents at the end of the 300 ms +20 mV depolarization (■, n=9). Data is normalised to the current elicited following the -100 mV preconditioning pulse and fitted to single or double component Boltzmann distributions (see text). The dotted lines represent zero current.

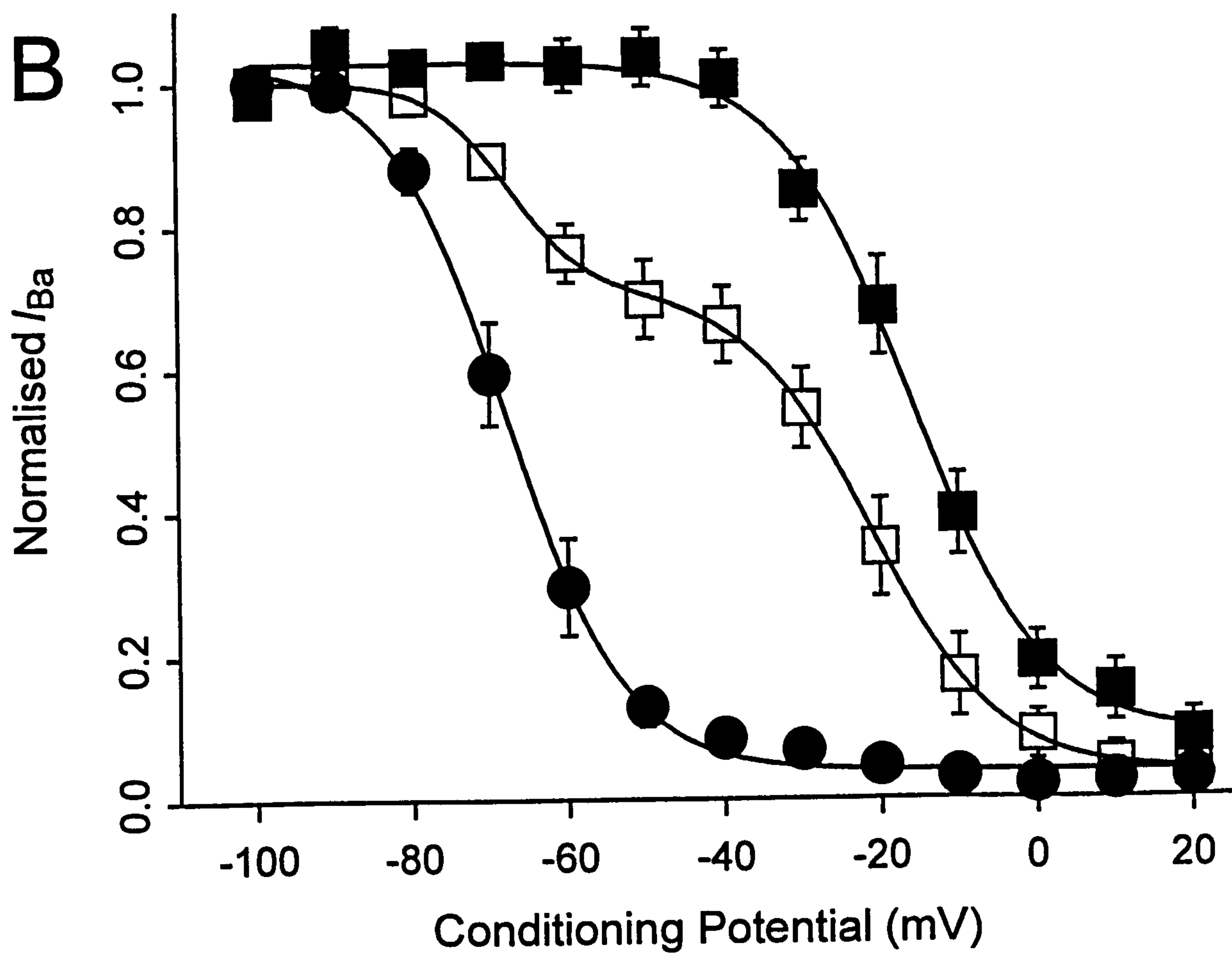
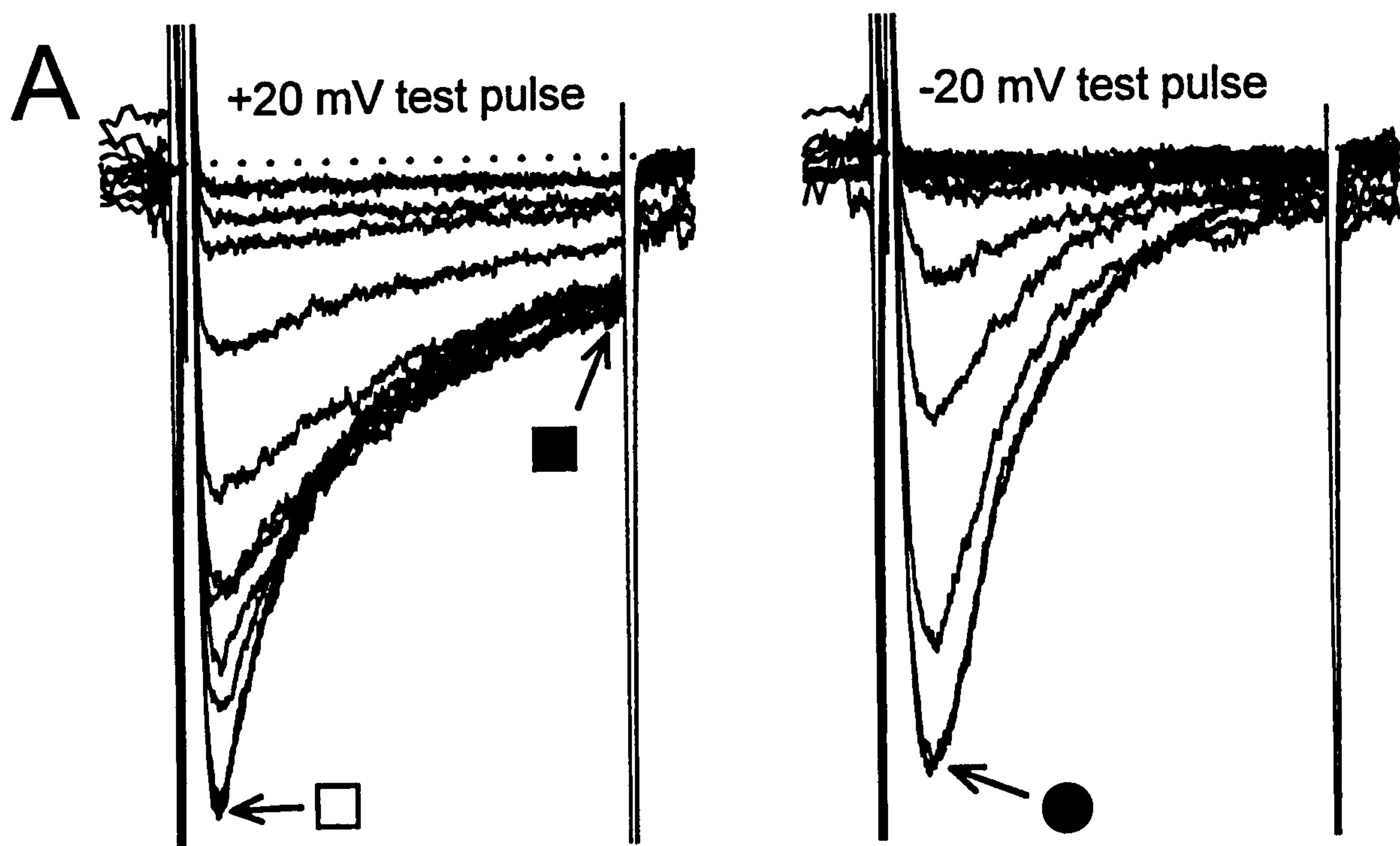
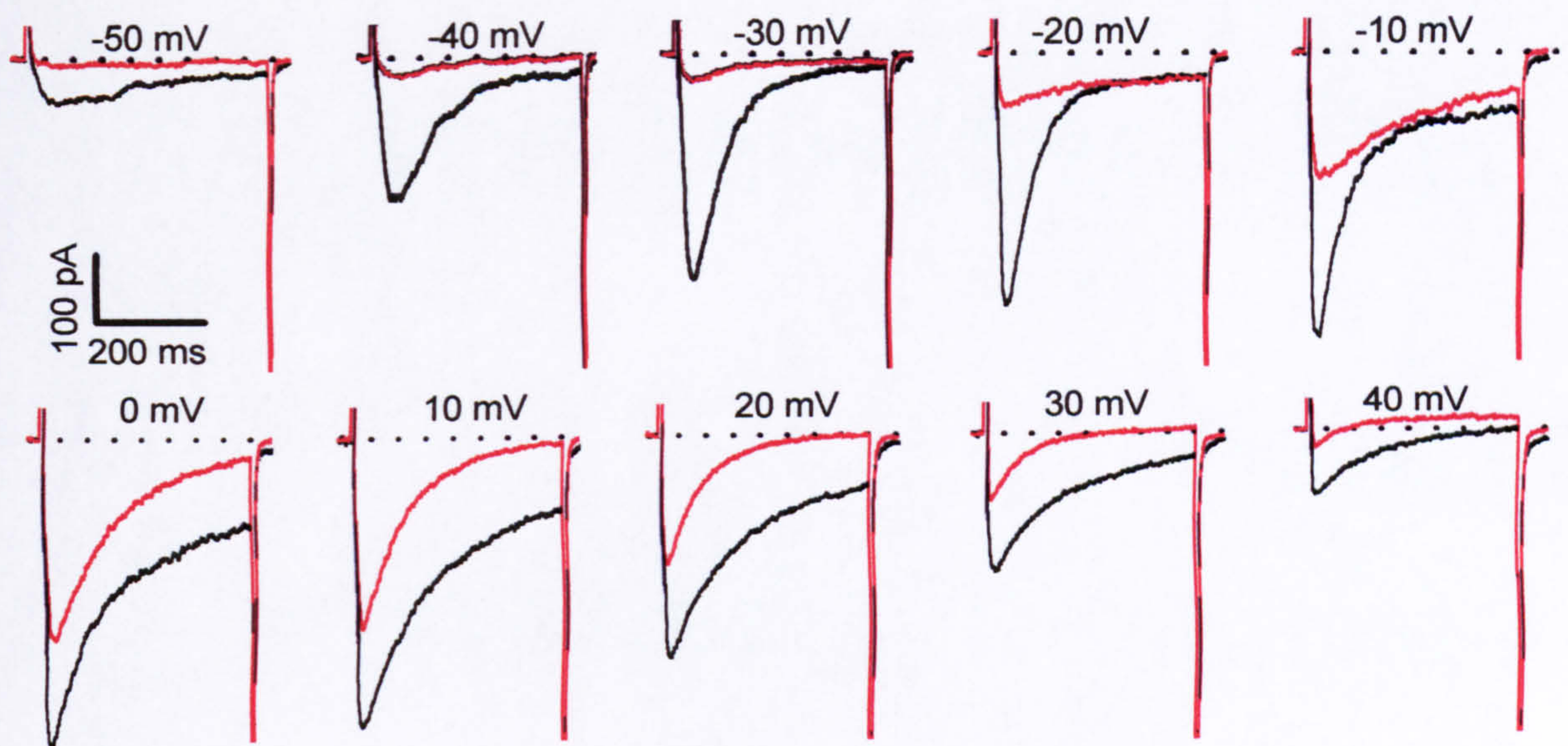


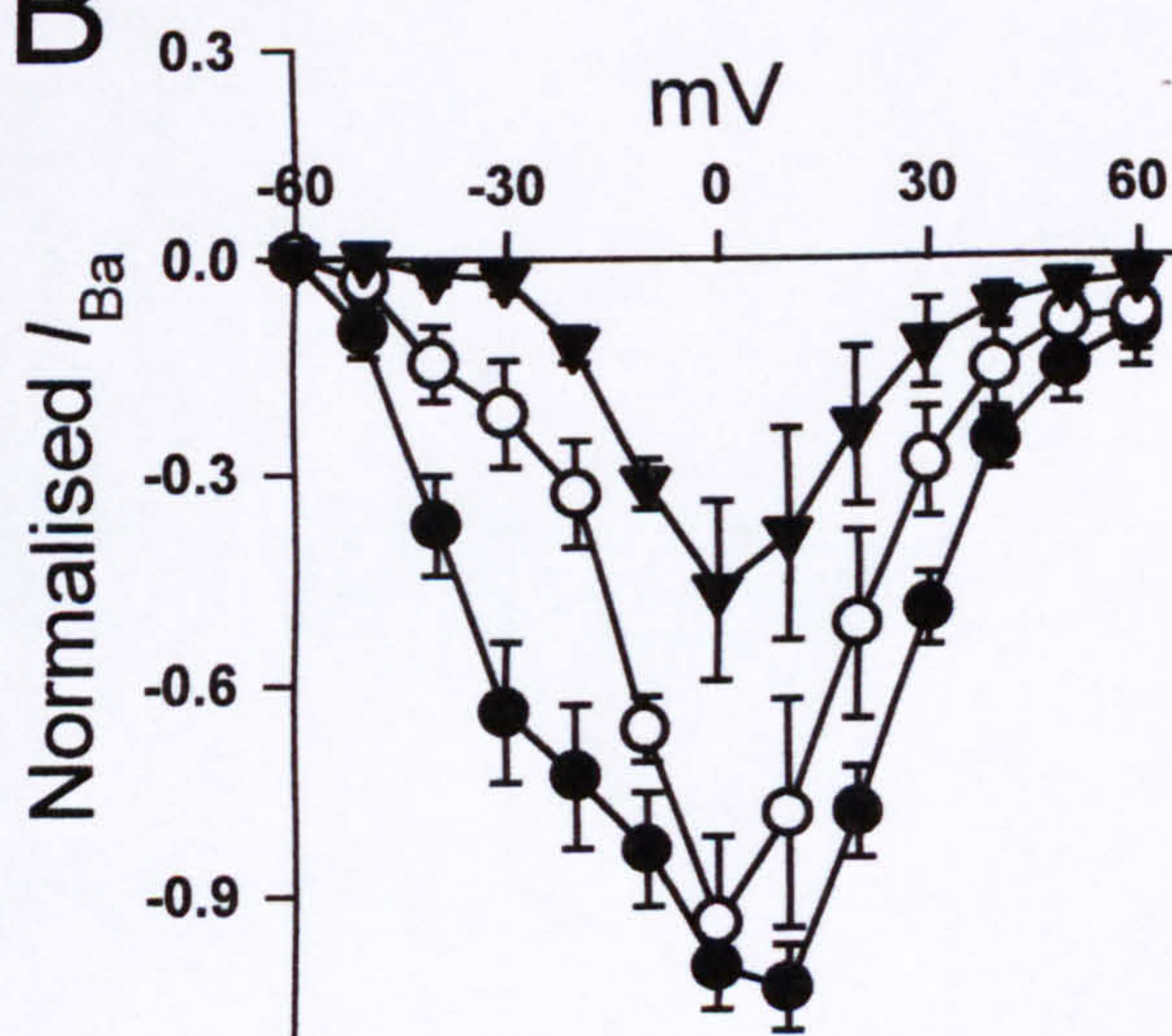
FIG 3.4. EFFECTS OF MIBEFRADIL ON L- AND T-COMPONENTS OF I_{Ba} .

A: 300 ms currents at a range of membrane potentials from V_H -80 mV in the absence (black traces) and presence (red traces) of 1 μ M mibefradil. The dotted lines represent zero current. **B & C:** Current voltage relationships at -80 mV, showing the current in PSS (●) and the effect of 0.1 (○) and 1 μ M mibefradil (▼) on current at peak (**B**, 30-50 ms) and end of pulse (**C**, 275-300 ms) in 4 cells, plotting the mean and S.E.M. of measurements, normalised to 0 mV in the absence of mibefradil. **D:** The effect of changing holding potential on block of I_{Ba} by mibefradil. From left to right: 200 ms current at +20 mV from V_H -50 mV; block by 1 μ M mibefradil (mib) at +20 mV from V_H -50 mV; partial recovery from block in continued presence of mib at +20 mV when the V_H was changed to -80 mV; return of complete block when the V_H was returned to -50 mV.

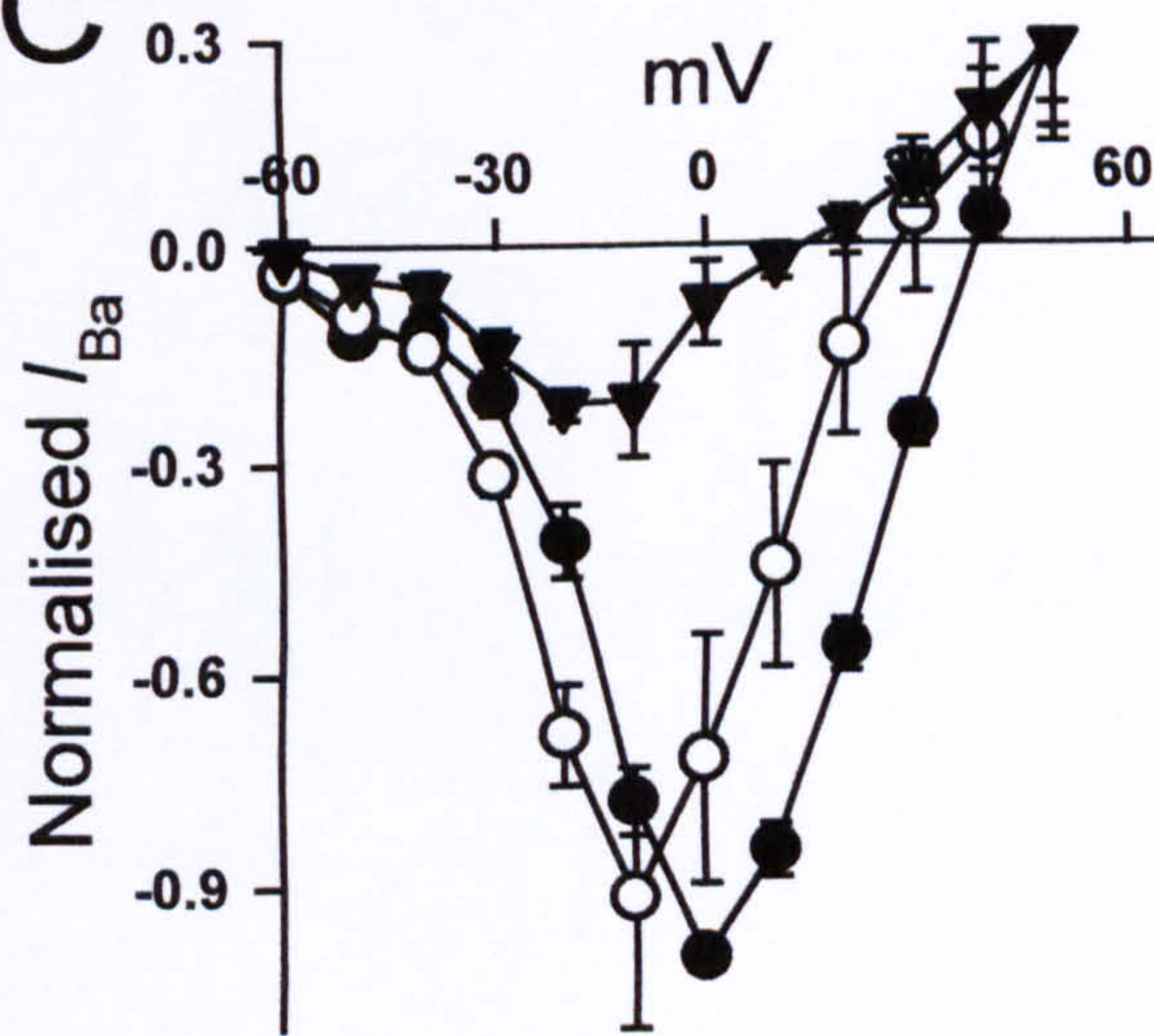
A



B



C



D

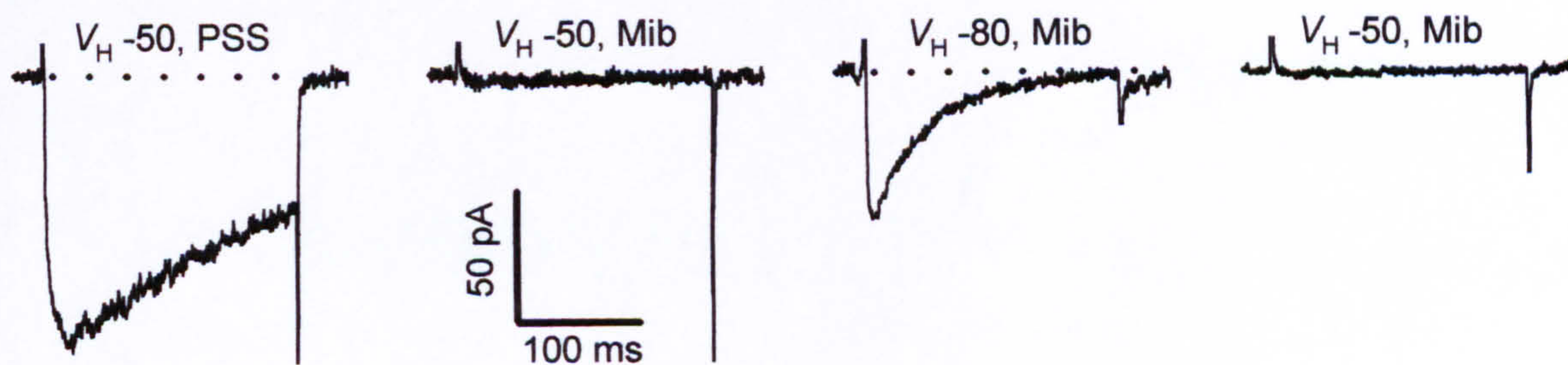
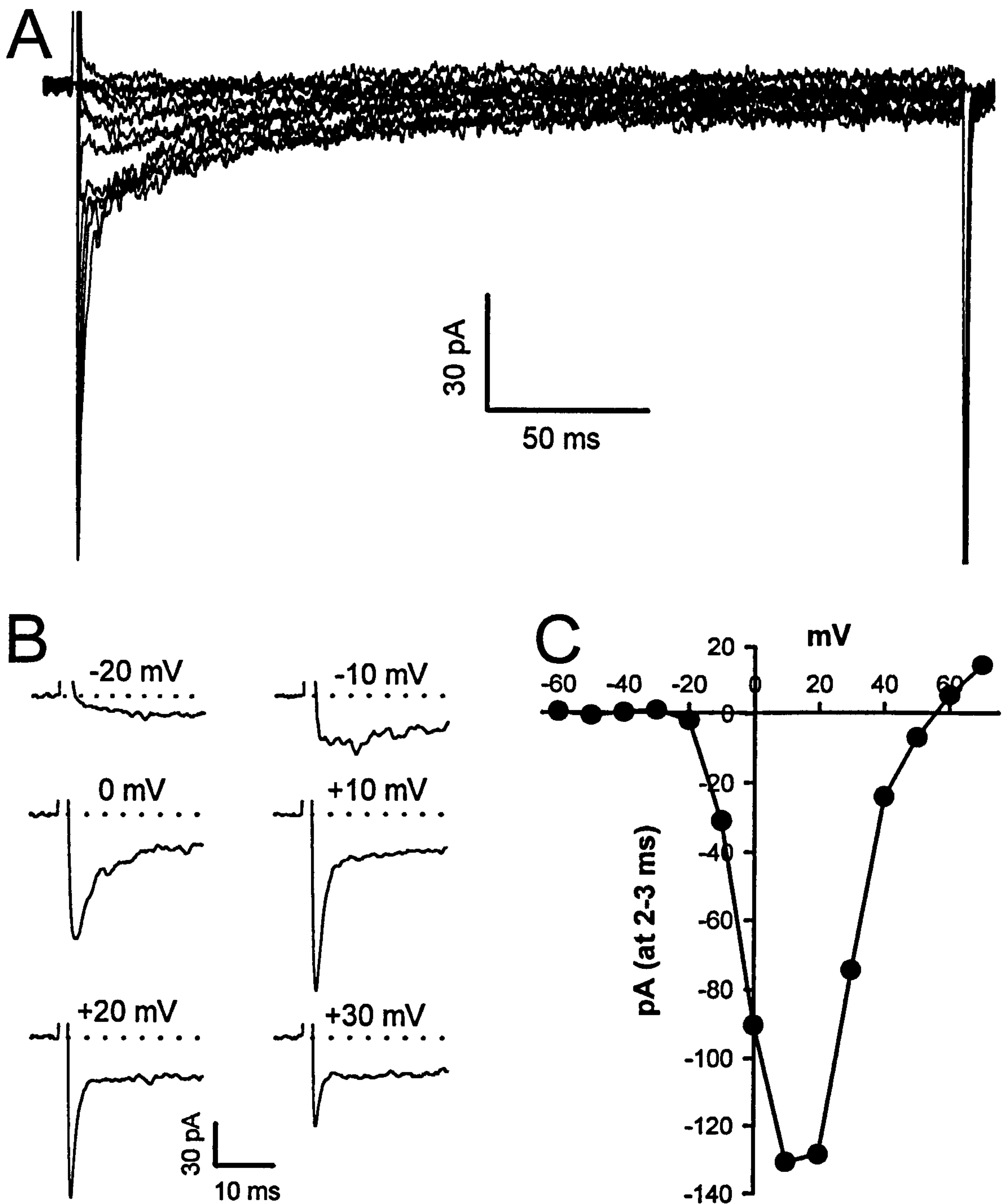


Fig. 3.5. Na^+ current in HMSMCs



Example of current voltage relationship of fast inward current (I_{Na}). **A:** 300 ms currents at potentials between -60 and +70 mV from V_{H} -50 mV. **B:** Some of the same traces as in A, showing only the first 20 ms. Dotted lines represent zero current. **C:** Peak measurements (2-3 ms) of fast component, plotted against membrane potential.

FIG. 3.6. OSCILLATING AND NON-OSCILLATING INWARD CURRENT IN HMSMCS

Examples of the two ways in which the amplitude of I_{Ca} and I_{Ba} (measured as integrated current) may change with time, recorded at 0 mV from V_H -60 mV, once every 10 seconds. In both cells the period of current run-up is not shown.

A: In this cell (with 1.5 Ca^{2+} in the bath solution), a steady non-oscillating current occurs. **B:** In a different cell (with Ba^{2+} in the bath solution), a spontaneous oscillating current occurs. The insert shows currents representative of the peak (p) and trough (t) of an oscillation (indicated by arrows). The dotted line represents zero current. In this cell, though not in all, the oscillations began almost immediately after recording began (see text). Not all cells with I_{Ca} were steady and not all cells with I_{Ba} demonstrated oscillations.

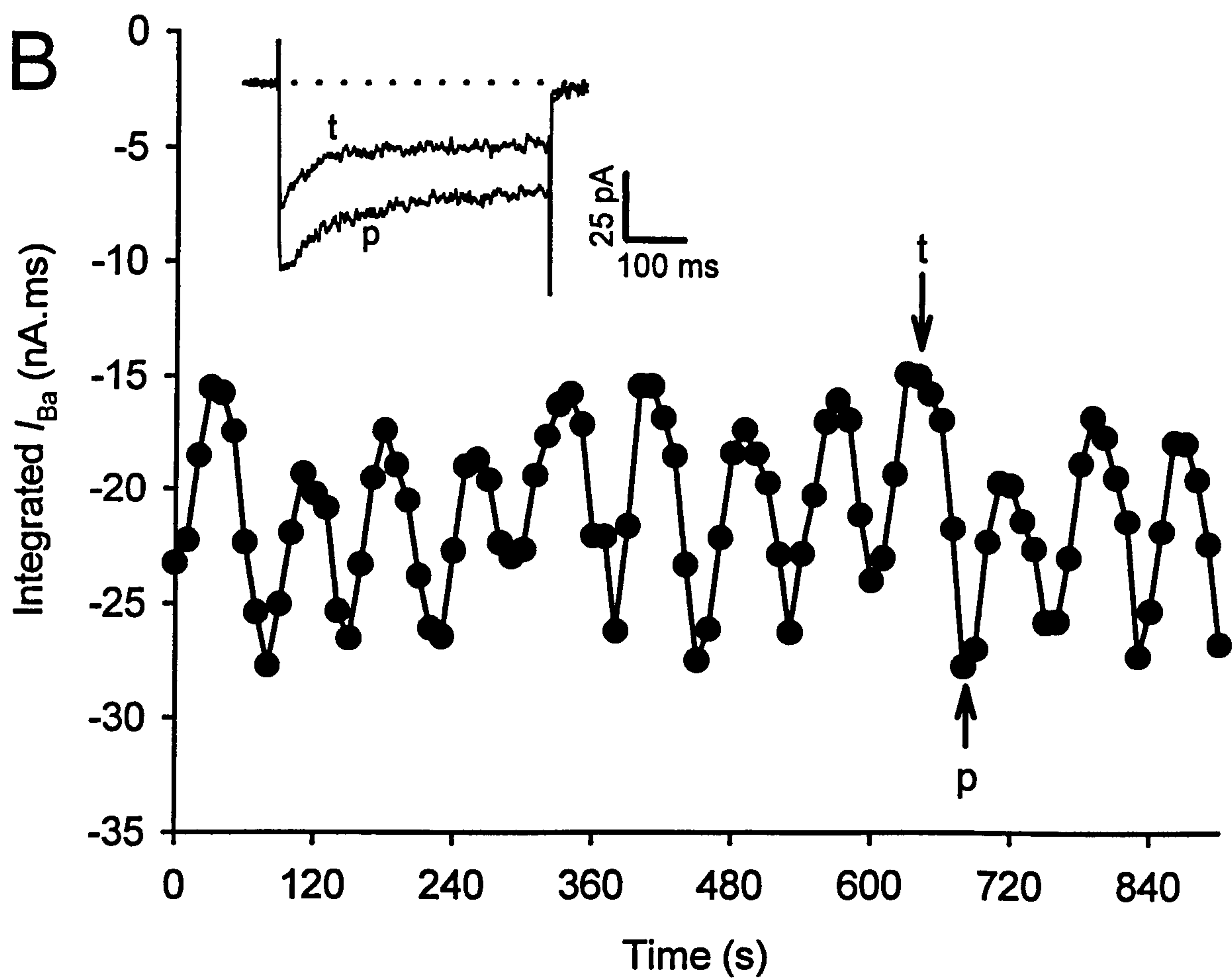
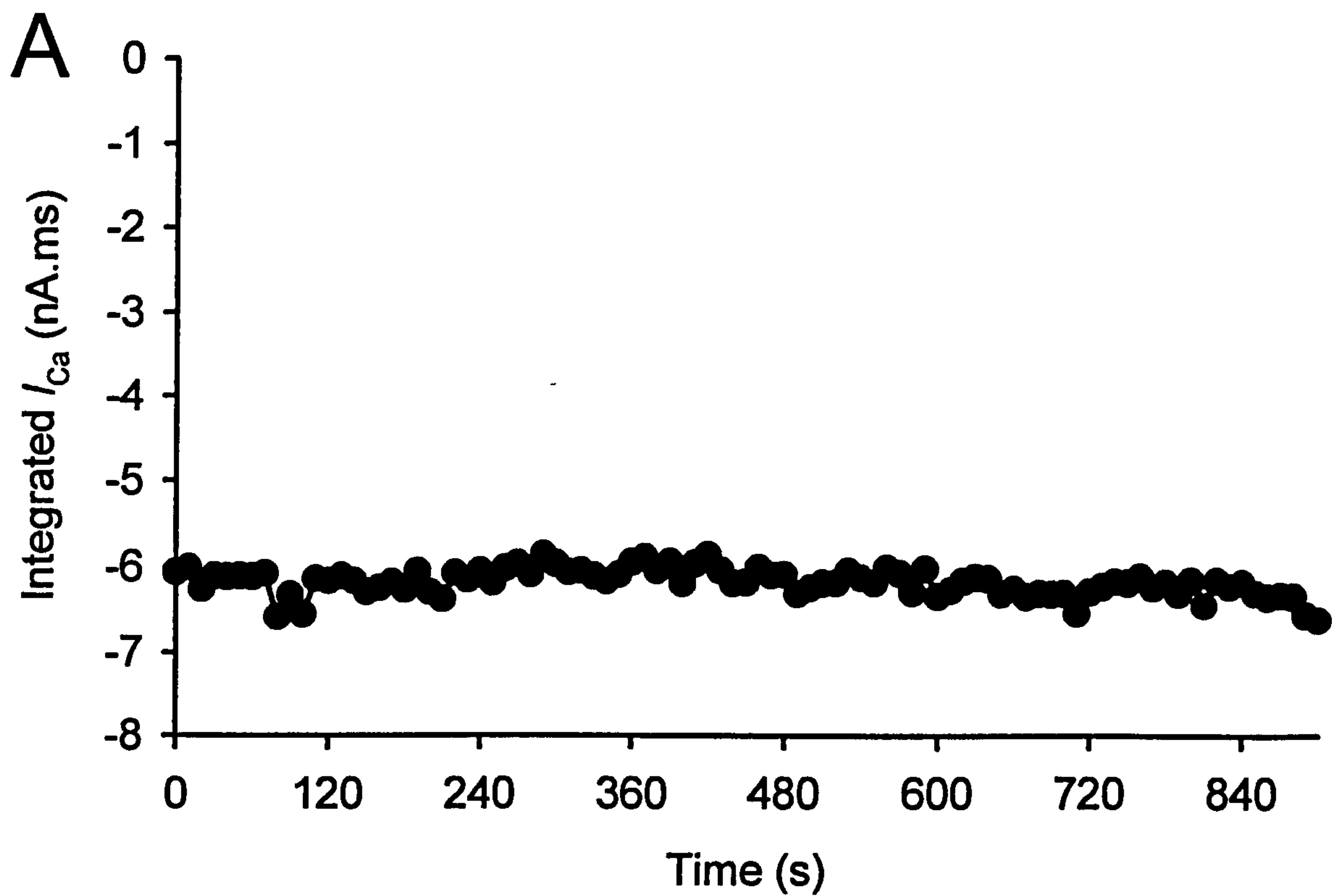
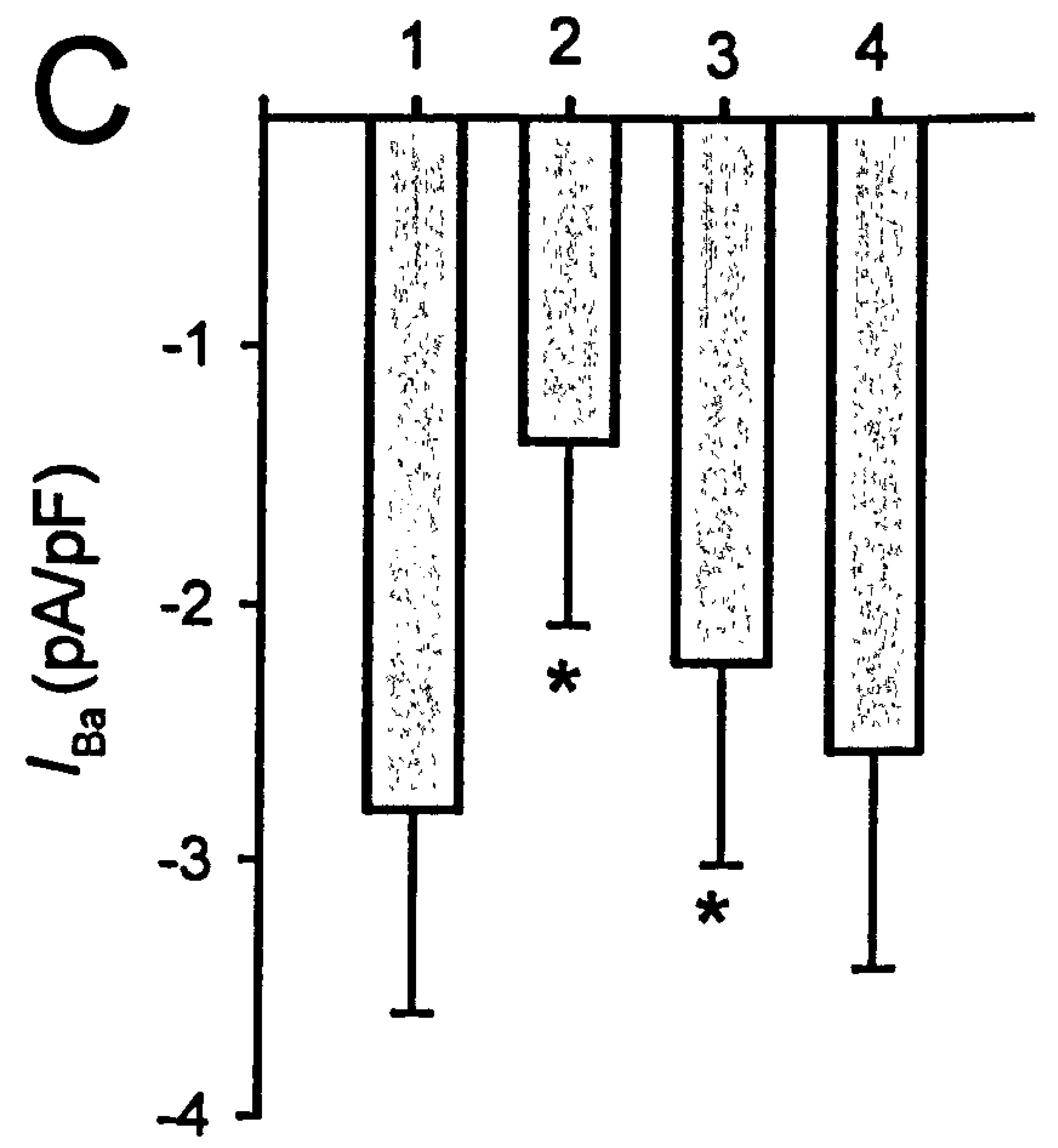
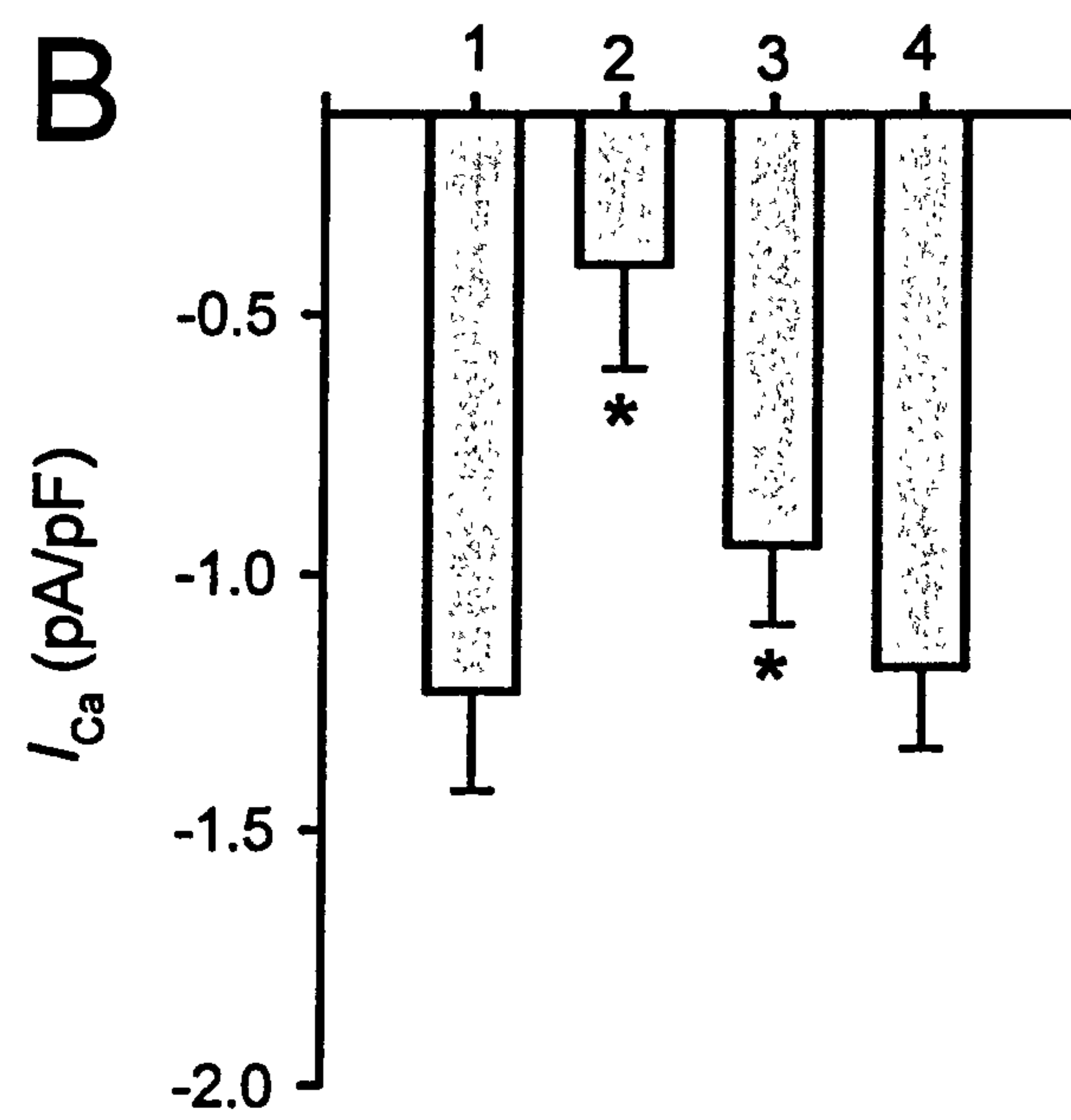
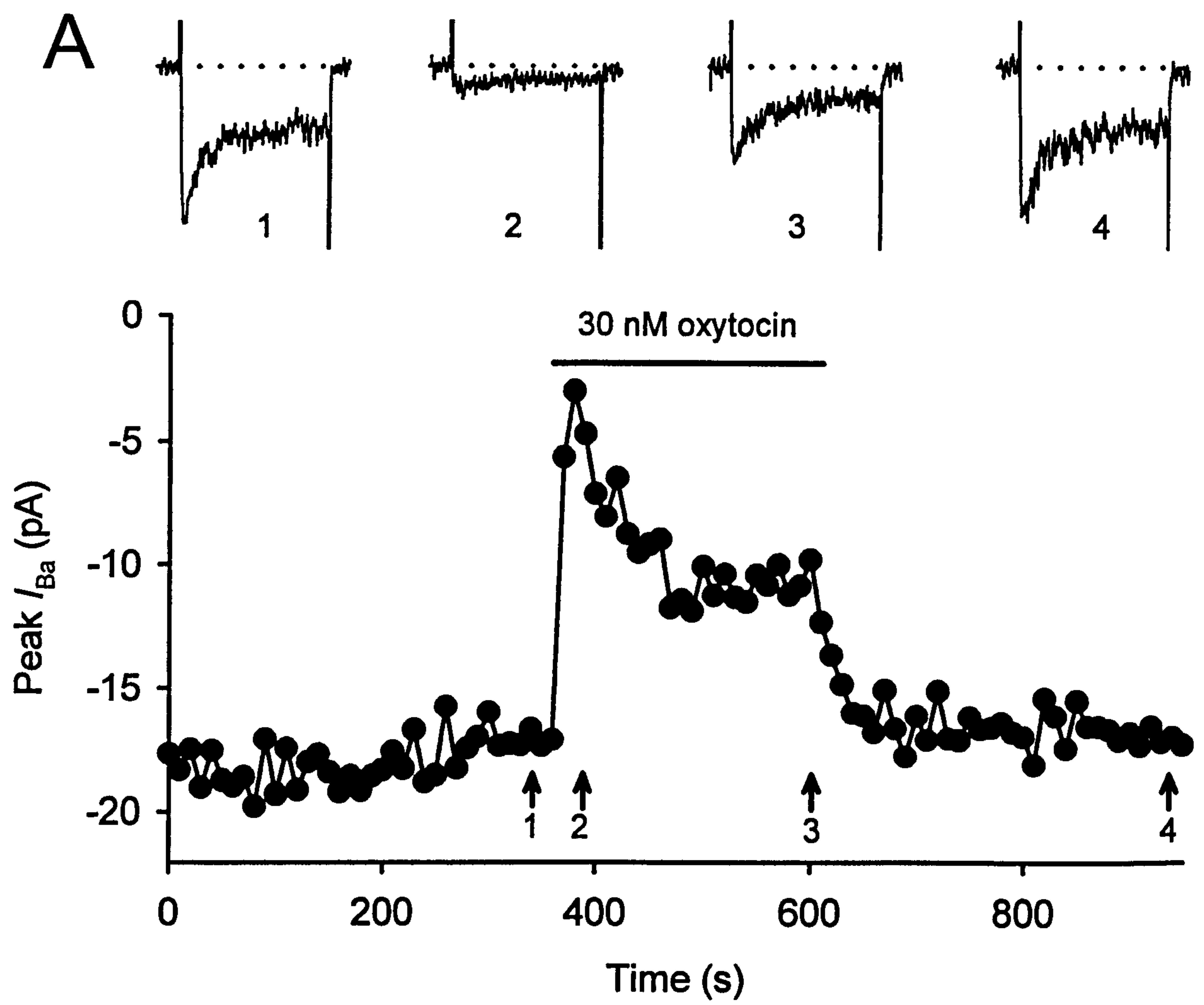


FIG 3.7. EFFECT OF OXYTOCIN ON I_{Ba} AND I_{Ca} IN HMSMCS

A: Example of the effect of oxytocin (OT) on I_{Ba} in a representative HMSMC. The arrows point to the measurements of representative current traces at each time point that were taken for statistical analysis: 1 = control period, 2 = transient component of inhibition, 3 = sustained component of inhibition and 4 = washout. The dotted lines represent zero current. The mean and S.E.M. of current densities from each of these four time points are plotted in **B** (I_{Ca} , $n = 4$) and **C** (I_{Ba} , $n = 5$). The asterisks indicate significant inhibition of current with respect to the control period ($P < 0.05$ by paired t-test).



3.2. DISCUSSION

3.2.1. L- AND T-TYPE Ca^{2+} CHANNEL CURRENTS

L-type Ca^{2+} channel current is abundant in HMSMCs. This is demonstrated by the effect of replacing Ca^{2+} with Ba^{2+} in the bath solution. The two effects of Ba^{2+} , namely increasing current amplitude several-fold and decreasing of current inactivation rate confirm the earlier work of Jmari *et al.* (1986) who, using sucrose gap in strips of pregnant rat myometrium, first demonstrated the voltage- and Ca^{2+} -sensitive components of L-type Ca^{2+} current inactivation. The very high sensitivity of current (recorded from V_H -50 mV) to micromolar or even sub-micromolar concentrations of CdCl_2 is also characteristic of L-type Ca^{2+} current (Amedee *et al.*, 1987; Honore *et al.*, 1989). The effect of Cd^{2+} on the T-component of I_{Ba} was not determined in the present study, although in non-pregnant rat it has been shown to be more sensitive to Ni^{2+} than to Cd^{2+} (Rendt *et al.*, 1992).

Electrophysiological and pharmacological analysis of I_{Ba} confirm earlier reports (Inoue *et al.*, 1990; Young *et al.*, 1991; Young *et al.*, 1993) of both L-type and T-type components in HMSMCs. The L-component demonstrated characteristics of a high threshold current, activating at potentials close to -40 mV and inactivating in a positive range of potentials ($V_{0.5} = -16$ mV). The T-component by contrast was a low threshold current (activating at potentials close to -60 mV and inactivating in a negative range of potentials: $V_{0.5} = -65$ mV). The $V_{0.5}$ values for L- and T-components were similar to those presented by Young *et al.*, (1993) in pregnant human myometrium (-27 mV for L-type and -70 mV for T-type), and by Mehrke *et al.*, (1994) for L- and T-type currents in $\alpha_{1\text{Cb}}$ -transfected Chinese hamster ovary cells (CHO) and human medullary thyroid carcinoma cells (h-MTC), respectively ($V_{0.5} = -15$ mV for L-type and -45 mV for T-type). Inactivation time constants for L- and T-components (211 ms at +20 mV for L-component and 37 ms at -20 mV for

T-component) were also similar to those described for L- and T- currents by Mehrke et al (1994) (416 ms at +20 mV for L-type and 33 ms at -10 mV for T-type).

The T-component at its potential of peak activation (-20 mV) was on average larger than the L-component at its potential of peak activation (+10 mV). This agrees with both previous reports (Inoue *et al.*, 1990; Young *et al.*, 1993). In fact, in Young *et al.* (1993) the T-component was 3-4 fold larger than the L-component.

3.2.2. EFFECT OF MIBEFRADIL ON T- AND L-COMPONENTS OF I_{Ba}

T- and L-components of I_{Ba} were also distinguished by their sensitivity to the Ca^{2+} channel antagonist mibefradil. As shown in Fig 3.4. B, when peak I_{Ba} was measured, mibefradil was more potent at blocking the T-component (step to -20 or -30 mV from V_H -80 mV) than the L-component (step to +10 mV or +20 mV from V_H -80 mV) at 0.1 and 1 μ M. When measured at the end of the pulse, however (Fig 3.4.C), where the current was mostly L-component, 0.1 μ M mibefradil produced a small but significant leftward shift in the current-voltage relationship, such that the current was enhanced at -20 mV but inhibited at +20 mV.

1 μ M mibefradil also enhanced L-component current decay at positive potentials (+10 to +40 mV) but not at negative potentials (-20 & -10 mV). These effects suggest that block of the L-type component of I_{Ba} may be voltage-dependent. This was confirmed by the effect of changing the holding potential on block of the L-component at +20 mV by 1 μ M mibefradil, where block was complete when V_H was -50 mV and recovered partially when V_H was lowered to -80 mV. Although similar changes in holding potential were not performed to determine whether block of the T-component was voltage-dependent, the T-component was blocked equally well at all potentials where it was the predominant

component of I_{Ba} (ie -50, -40, -30 and -20 mV), suggesting that its block was not voltage-dependent.

Voltage-dependent block of L-type Ca^{2+} current by mibefradil also occurred in h-MTC cells where the EC_{50} was 18 and 3 μM from V_H -80 and -50 mV, respectively (Merhke *et al.*, 1994) and in guinea-pig cardiac myocytes where it was 12 μM from V_H -80 mV and was 0.23 μM from V_H -50 mV (Liang-min & Osterrieder, 1991). In contrast, T-channel block in human medullary thyroid carcinoma cells (h-MTC cells) was not voltage-dependent (Merhke *et al.*, 1994). The sensitivity of the T-component to mibefradil in the present study is also similar to the T-current in rat azygos vein smooth muscle cells (Mishra & Hermsmeyer, 1994a), however, the L-type current in azygos vein SMCs differed in its sensitivity to mibefradil from both the present study and the above described h-MTC cells and guinea-pig cardiac myocytes. Block of the L-current in the azygos vein was neither voltage-dependent (block by 1 μM mibefradil was similar from V_H -80 mV and V_H -30 mV) nor use-dependent (indicative of open channel block) but was characteristic of resting state block only (Mishra & Hermsmeyer, 1994b). Also in the azygos vein, decay of L-type current was not affected by mibefradil at either holding potential. The voltage-dependence and leftward shift in the activation of the L-component by mibefradil in HMSMCs resembles the partially agonistic properties of some dihydropyridine Ca^{2+} channel antagonists (eg nifedipine) (Aaronson *et al.*, 1988). This is unusual since mibefradil has been shown to bind preferentially to the desmethoxyverapamil binding site in cardiac membranes rather than the dihydropyridine binding site (Liang-min & Osterrieder, 1991), despite the fact that verapamil blocks L-type Ca^{2+} channels in a use-dependent rather than voltage-dependent manner (see chapter 4 for further discussion on the three major classes of Ca^{2+} antagonist).

3.2.3. OTHER FAST INWARD CURRENT IN HMSMCS

A very fast voltage-gated inward current, probably a sodium current (I_{Na}), was present in a very small proportion of cells (5 out of more than a hundred). The threshold and potential of peak activation of this current were similar to both the L-component of I_{Ba} and the I_{Na} described in the pregnant rat myometrium (Yoshino *et al.*, 1997), although the speed of decay at +10 mV was slower (2 ms) than in the rat (0.5 ms at +10 mV) and the inactivation occurred over a more positive range of potentials ($V_{0.5} = -31$ mV) than in the rat (-59 mV, Yoshino *et al.*, 1997). The cell capacitance of these 5 cells (25 ± 6 pF) was below average for pregnant human myometrium (98.7 pF, see chapter 2) so it is possible that these cells represent a morphologically distinct population.

3.2.4. PHYSIOLOGICAL RELEVANCE OF I_{Ca} AND I_{Na} IN HUMAN MYOMETRIUM

The L-type Ca^{2+} channel is ubiquitous in the uterus of all mammalian species that have been studied, and many *in vitro* functional studies using three classes of L-type selective Ca^{2+} channel antagonist have demonstrated that Ca^{2+} influx through L-type Ca^{2+} channels is essential for spontaneous and agonist-induced contraction of pregnant or non-pregnant myometrium in several species, including humans and rats (see Table 1.4.). The density of I_{Ca} in rat myometrium increases during the first trimester of pregnancy and then decreases slowly towards term (Yoshino *et al.*, 1997) despite a 7-fold increase in α_1 subunit mRNA expression at term compared to early pregnancy (Mershon *et al.*, 1994; Tezuka *et al.*, 1995). In pregnant pigs also, there is a 2-3 fold increase in I_{Ba} with respect to non-pregnant (ZhuGe & Hsue, 1994). It is not known whether expression of α_1 mRNA or density of I_{Ca} changes during pregnancy or in labour in the human myometrium.

Selective inhibition of fast Na^+ current with TTX either in rats or humans in contrast, was unable to inhibit spontaneous or agonist-induced myometrial contractions *in vitro* (Poli *et*

al., 1990; Phillippe & Basa, 1997a). Alternatively, $\text{Na}_v2.3$ channel protein has also been co-localised with connexin 43 gap junction protein in the pregnant mouse myometrium (Knittle *et al.*, 1996), suggesting an alternative function for I_{Na} in conduction of electrical activity through the tissue, such that I_{Na} may influence co-ordinated contraction of the entire uterus *in vivo* rather than force, duration or frequency of contractions *in vitro*.

A possible explanation for the lack of involvement of I_{Na} in myometrial contractility *in vitro* lies in its electrophysiological properties. The negative inactivation (-59 mV) but positive activation threshold (below -50 mV and -30 mV) (Ohya & Sperelakis, 1989; Inoue & Sperelakis, 1991; Yoshino *et al.*, 1997), means that currents induced at the range of membrane potentials at which activation and inactivation overlap (also known as the window current) will be very small. This contrasts with the L-type I_{Ca} which activates and inactivates in a more similar range of membrane potentials, resulting in a larger window current. The resting membrane potential of the myometrium, which is more positive than in vascular smooth muscle, cardiac myocytes and neurones will result in tonic inactivation of a much larger proportion of Na^+ channels than of L-type Ca^{2+} channels, so that a much smaller proportion of Na^+ channels will be available to open during depolarisations. It is therefore puzzling why the expression of $\text{Na}_v2.3$ mRNA in mouse (Knittle *et al.*, 1996) and I_{Na} density in rat (Inoue & Sperelakis, 1991; Yoshino *et al.*, 1997) should so clearly increase during gestation in the rat myometrium until at term it is the dominant current, and then decline post-partum.

The predominance of a T-current in human myometrium and its apparent absence in the majority of studies in rats (Ohya & Sperelakis, 1989; Miyoshi *et al.*, 1991; Inoue & Sperelakis, 1991; Yoshino *et al.*, 1997), with one exception (Rendt *et al.*, 1992), is also difficult to explain. The existence of T-component in non-pregnant HMSMCs was not investigated so it is not known whether expression of this channel changes during pregnancy as do I_{Na} and the L-component of I_{Ca} in the rat. These differences in channel

expression between rat and human may be influenced by regions of the uterus from which cells were taken in the different studies, a fact that will be complicated by the basic anatomical differences between rat and human uteri (Devedeux *et al.*, 1993). In the human myometrium, for example, currents were only recorded in cells obtained from lower segment (isthmus) and not from the main body of the uterus (fundus). It is not known whether myocytes from the fundus in humans express more I_{Na} than in myocytes from the lower segment.

A physiological role for T-type Ca^{2+} channels in action potential generation and contraction in human myometrium remains to be determined. This contrasts particularly with the cardiac T channel, which because of its concentration in the sino-atrial node and Purkinje fibres of the heart, the very negative resting membrane potential in the myocardium (~ -80 mV), and the negative chronotropic effects of mibefradil, is likely to be involved in cardiac pacemaker activity (Triggle, 1998). Although the likelihood of T-channel involvement in contraction is reduced by its negative inactivation threshold, its unusually large current density with respect to the L-component may mean that a large enough number of channels will still be available to open at the resting membrane potential. This, together with its low threshold of activation, resulting in a significant window current, albeit over a more negative range of membrane potentials than the L-current, may be enough to influence slow wave or action potential generation. The effects, if any, of mibefradil on membrane potential and myometrial contractility have not been determined.

3.2.5. INHIBITION OF Ca^{2+} CHANNEL CURRENTS BY OXYTOCIN

Oxytocin is a uterotonic hormone that is present in the plasma throughout pregnancy and is involved in mediating labour and parturition. The principle function of OT in this process is to promote myometrial contraction and part of this action clearly involves opening of non-selective cation and Ca^{2+} -activated Cl^- channels, which probably account

for the plateau-type action potentials often induced by OT (Kuriyama & Suzuki, 1976b; Kawarabayashi *et al.*, 1990; Nakao *et al.*, 1997; Arnaudeau *et al.*, 1994; Shimamura *et al.*, 1994b). The most extensively documented action of OT in the myometrium, is arguably its effect on release of Ca^{2+} from intracellular stores via IP_3 production (Marc *et al.*, 1986; Schrey *et al.* 1987; Coleman, *et al.*, 1988; Anwer & Sanborn, 1989; Zhuge *et al.*, 1995). Since OT stimulates contraction therefore, the inhibition of I_{Ba} and I_{Ca} in the present study is apparently contradictory. It is in agreement however with a similar study in rat (Inoue *et al.*, 1992) where it inhibits I_{Ca} , but in disagreement with two studies in pigs (ZhuGe & Hsu, 1994; ZhuGe *et al.*, 1995) where it enhances I_{Ca} . If this inhibition is genuine, a possible explanation is an indirect effect on the Ca^{2+} channel via release of Ca^{2+} from internal stores, since it is known that I_{Ca} is inhibited by intracellular Ca^{2+} (Jmari *et al.*, 1986). This may also explain the transient nature of the inhibition, since released Ca^{2+} may be bound by the dialysed EGTA, taken back up into the stores or pumped out of the cell. This idea is however complicated by the fact that I_{Ba} is inhibited as well as I_{Ca} suggesting that stored Ca^{2+} may remain in the cell even after prolonged replacement of external Ca^{2+} with Ba^{2+} . It is unlikely that Ba^{2+} may in fact replace Ca^{2+} in the stores, since release of Ba^{2+} from stores would not inhibit I_{Ba} (inactivation of the channel is only sensitive to $[\text{Ca}^{2+}]_i$ not $[\text{Ba}^{2+}]_i$) (Ganitkevitch *et al.*, 1987).

It is also unclear how quickly one would expect Ca^{2+} released from stores to be buffered by the 10 mM EGTA in the pipette and how this would be affected by cell size, intracellular store structure, proximity of the stores with the plasma membrane and the involvement of endogenous buffers. If the inhibition by OT is not indirect via release of Ca^{2+} then the only other likely explanation is a direct action of OT on the channel via transduction from its receptor to a G protein. Indeed, there is recent evidence that voltage-gated Ca^{2+} channels may be inhibited (neuronal non-L-type) or activated (vascular L-type) by G protein $\beta\gamma$ subunits (Dolphin, 1998; Viard *et al.*, 1999).

3.2.6. OSCILLATIONS OF I_{Ca} AND I_{Ba}

The spontaneous and OT-induced rhythmic oscillation of both I_{Ca} and I_{Ba} in a proportion of HMSMCs is an unusual phenomenon. These currents were recorded under normal voltage-clamp and induced by brief depolarisations to -10 or 0 mV, so they probably represent movement of ions through L-type Ca^{2+} channels as described above. A possible explanation for these oscillations lies in the oscillations in intracellular Ca^{2+} that are known to occur spontaneously in the myometrium or in response to oxytocin stimulation (Thornton *et al.*, 1992b; Morgan *et al.*, 1993b; Lynn *et al.*, 1993; Phillippe & Basa, 1997a), since L-type Ca^{2+} current is sensitive to $[Ca^{2+}]_i$ (Ganitkevitch *et al.*, 1987). These oscillations are dependent on extracellular Ca^{2+} influx (Thornton *et al.*, 1992b; Phillippe & Basa, 1997a). The frequency of oscillations in I_{Ca} and I_{Ba} is in a similar order of magnitude (1-2 per min) to the above described oscillations in $[Ca^{2+}]_i$, suggesting that elevations in $[Ca^{2+}]_i$ may be responsible for inhibiting I_{Ca} or I_{Ba} .

A possible relationship between intracellular Ca^{2+} oscillations and spontaneous transient inward currents (STICs) has also been demonstrated in smooth muscle cells of guinea-pig trachea (Jansen & Sims, 1994). These transient currents, carried by Cl^- through Ca^{2+} -activated Cl^- channels, were very brief (less than 1 s duration) and occurred at negative membrane potentials where L-type Ca^{2+} channels will be in the resting state (-70 mV), but were enhanced by depolarisation-induced Ca^{2+} entry. In another similar study in ras-transformed 3T3 cells bradykinin-induced oscillations in I_{Ba} (frequency 0.1-0.05 Hz) are synchronous with $[Ca^{2+}]_i$ oscillations (Higashida *et al.*, 1992a). The current oscillations were however abolished, and no inward current resulted, when Ba^{2+} (50 mM) was replaced by Ca^{2+} or Sr^{2+} in the bath solution, suggesting that the oscillations were somehow Ba^{2+} -specific (Higashida *et al.*, 1992b). In addition, these oscillations were blocked by antagonists of receptor gated Ca^{2+} channels ruling out an involvement of L-type voltage-gated Ca^{2+} channels.

In the present study, the effect of CPA on I_{Ba} , even when cells were dialysed with 10 mM EGTA and external Ca^{2+} was replaced by Ba^{2+} , suggests that Ca^{2+} was still being released from the SR. In two out of a further three cells however, I_{Ba} oscillations still occurred even when extreme measures were taken to buffer $[Ca^{2+}]_i$ by dialysing cells with 30 mM EGTA and 20 mM BAPTA and heparin (to prevent Ca^{2+} release from stores). This may suggest that the oscillations in I_{Ca} and I_{Ba} are not due entirely to intracellular Ca^{2+} but that other intracellular mechanisms that modulate L-type channel activity, such as protein kinases or G protein $\beta\gamma$ -subunits (Beech & McHugh, 1996; Dolphin, 1998; Viard *et al.*, 1999), or some other yet unknown intrinsic rhythmicity of the channel gating mechanism itself, may be involved. The physiological relevance of these oscillations with regards to action potential generation or the contractile rhythmicity of the myometrium, if any, is unknown.

Chapter 4

**Ca²⁺ Channel Antagonist Properties of
the Cyclooxygenase-2 Inhibitor
Nimesulide in HMSMCs of Pregnant
Women**

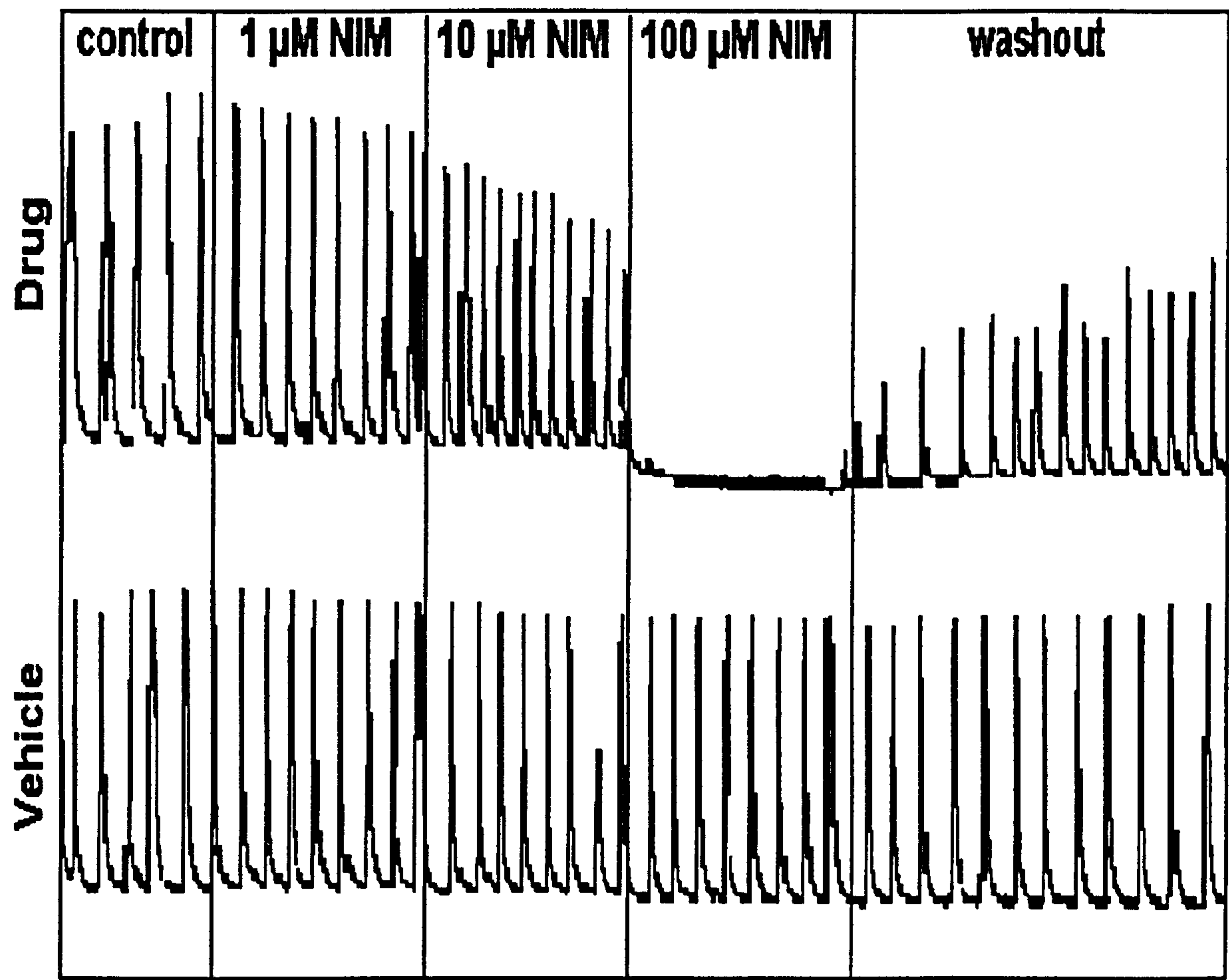
4.1. METHODS AND RESULTS

4.1.1. EFFECT OF NIMESULIDE AND INDOMETHACIN ON MYOMETRIAL CONTRACTILITY

All experiments in this chapter were performed with freshly dispersed HMSMCs from pregnant women at term who were not in active labour. Investigation of the effects of nimesulide and indomethacin on myometrial contraction was performed by Dr Robert Sawdy (Dpt. Obstetrics and Gynecology, Queen Charlotte's & Chelsea Hospital, London) and the methodology for these experiments is described in detail in Sawdy *et al.*, (1998). Briefly, strips of pregnant human myometrium were mounted under 3g of resting tension in an organ bath containing phosphate buffered saline (PBS) bubbled with 95 % O₂ and 5 % CO₂ at 37 °C, and isometric tension recorded. PBS contained in mM: NaCl, 119; KCl, 4.7, CaCl₂, 2.5; MgSO₄, 1.2; NaHCO₃, 25; Na₂PO₄, 1.2; EDTA, 0.03; glucose, 5.5, pH 7.4. Treatment and vehicle (DMSO, maximum 0.1 %) control experiments were run simultaneously and contractions in the treatment strip were normalised for spontaneous changes in the control strips. Three contraction parameters were analysed: 1) total work per treatment interval (summed area under all of the contractions during the interval), 2) mean work done per contraction (mean area under individual contractions during an interval), and 3) mean contraction amplitude during an interval.

Fig 4.1. illustrates an example of an experiment in which the effect on myometrial contractility of several concentrations of nimesulide was recorded. The lower trace shows the effect of increasing concentrations of vehicle (DMSO) alone, while the upper trace illustrates the relaxant effects of 1, 10, and 100 µM nimesulide. Fig 4.2. shows the concentration-dependency of the inhibitory effect of nimesulide on the three contraction parameters, which was similar for all three, being partial although not statistically significant at 10 µM, but nearly complete and highly significant at 100 µM.

FIG 4.1. EFFECT OF NIMESULIDE ON SPONTANEOUS CONTRACTIONS IN PREGNANT HUMAN MYOMETRIUM



Cumulative dose-response for nimesulide

Spontaneous isometric contractions in strips of pregnant human myometrium. **Upper trace:** the effect of 1, 10, and 100 μM nimesulide, applied at approximately 1 hr intervals, following a control period and followed by washout. **Lower trace:** simultaneous vehicle (DMSO) control experiment in another strip from the same myometrial sample, showing lack of effect of DMSO at the concentrations used to dissolve each concentration of nimesulide.

In similar experiments, indomethacin also suppressed myometrial contractions (example not shown), although over a somewhat higher concentration range. Indomethacin caused a significant block at 100 μM , and essentially abolished contractions at 300 μM . The concentration-dependency of the effect of indomethacin on the three indices of spontaneous activity is also shown in Figure 4.2.

4.1.2. INHIBITION OF Ca^{2+} CHANNEL CURRENT BY NIMESULIDE AND INDOMETHACIN

The somewhat rapid inhibition of spontaneous contractility by 100 μM nimesulide and 300 μM indomethacin, and the fact that the potency of indomethacin was approximately 3-fold less than that of nimesulide, suggests that the effects of these two cyclooxygenase inhibitors may not be solely through inhibition of endogenous prostaglandin production within the myometrium strips. The effects of the two drugs on calcium channel currents in single freshly dispersed HMSMCs was therefore investigated using the drug concentrations that completely inhibited spontaneous contractions.

For the recording of I_{Ca} and I_{Ba} , $\text{Ca}^{2+}/\text{Cs}^+/\text{TEA}$ or $\text{Ba}^{2+}/\text{Cs}^+/\text{TEA}$ bath solutions (Table 2.1.) and the Cs^+ -containing pipette solution (Table 2.2.) were used. Nimesulide (100 μM) or indomethacin (300 μM) was applied during repetitive 200 ms depolarizations to +10 mV applied at 0.1 Hz, from a holding potential of -50 mV, following a 2 min control period during which the current amplitude was stable. The integral of currents (measurement of area under the current trace) were calculated for current in the absence (control period) and presence of drug (2-3 min were allowed for the inhibitor to have its effect). The effects of both drugs on both I_{Ba} (10 mM Ba^{2+}) and I_{Ca} (1.5 mM Ca^{2+}) were assessed. Examples of the effects of 100 μM nimesulide and 300 μM indomethacin on I_{Ba} and I_{Ca} are shown in Fig 4.3. and the mean inhibition of integrated current by nimesulide

and indomethacin are shown in Fig 4.4A and 4.4B, respectively (inhibition was significant in all cases, $P < 0.05$). These experiments therefore show that 100 μM nimesulide and 300 μM indomethacin produce a similar degree of block to each other, but do not block I_{Ba} or I_{Ca} as much as they block contraction and that I_{Ba} and I_{Ca} are inhibited to a similar extent.

In order to determine whether the inhibition of Ca^{2+} channel current by nimesulide was at least in part due to an attenuation of intrinsic basal prostaglandin synthesis, the effect of 100 μM nimesulide on I_{Ba} was also recorded when the drug was applied after a 3 min pre-incubation in (and in the continuing presence of) either 30 μM prostaglandin E_2 (PGE_2) or prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$). As is illustrated in Fig 4.4C, the extent of I_{Ba} inhibition in the presence of either prostaglandin was similar to that observed in the absence of prostaglandin (shown in Fig 4.4.A).

To ensure that current inhibition was not due to the DMSO vehicle, the effect of the highest concentration of DMSO used (0.1%) on I_{Ba} was also examined in 9 cells. Current was not significantly affected (integral current corrected for cell size $407 \pm 64 \text{ pA ms pF}^{-1}$ vs 446 ± 79 in PSS). In addition, experiments were performed to determine whether current inhibition by 100 μM nimesulide and 0.1 % DMSO was affected by the presence or absence of DMSO in the control period before nimesulide (dissolved in DMSO) was added, in order to separate the effect of nimesulide *per se* from the effect of DMSO. The inhibition was similar in both cases ($39 \pm 3\%$, $n = 9$ vs. $41 \pm 3\%$ $n = 11$, respectively).

This apparent Ca^{2+} antagonistic property of the two cyclooxygenase inhibitors, especially nimesulide, was considered of sufficient interest that further experiments were performed to determine the mechanisms of I_{Ba} block by nimesulide, whether the T-component of I_{Ba} was similarly blocked and whether the potency of block was affected by pH.

FIG. 4.2. INHIBITION OF SPONTANEOUS MYOMETRIAL CONTRACTIONS BY NIMESULIDE AND INDOMETHACIN

Concentration-dependency of the inhibition of myometrial contractility by nimesulide (●) and indomethacin (○). **A:** total work done per treatment interval, **B:** work done per contraction, **C:** peak contraction amplitude. Values are shown as mean \pm S.E.M. Each concentration of nimesulide was applied to 14 strips of myometrium (from 7 biopsies). 10 μ M indomethacin was applied to 23 strips from 11 biopsies, 100 μ M indomethacin was applied to 25 strips from 13 biopsies, 300 μ M indomethacin was applied to 7 strips from 3 biopsies. Asterisks indicate significant inhibition by drug ($P < 0.05$).

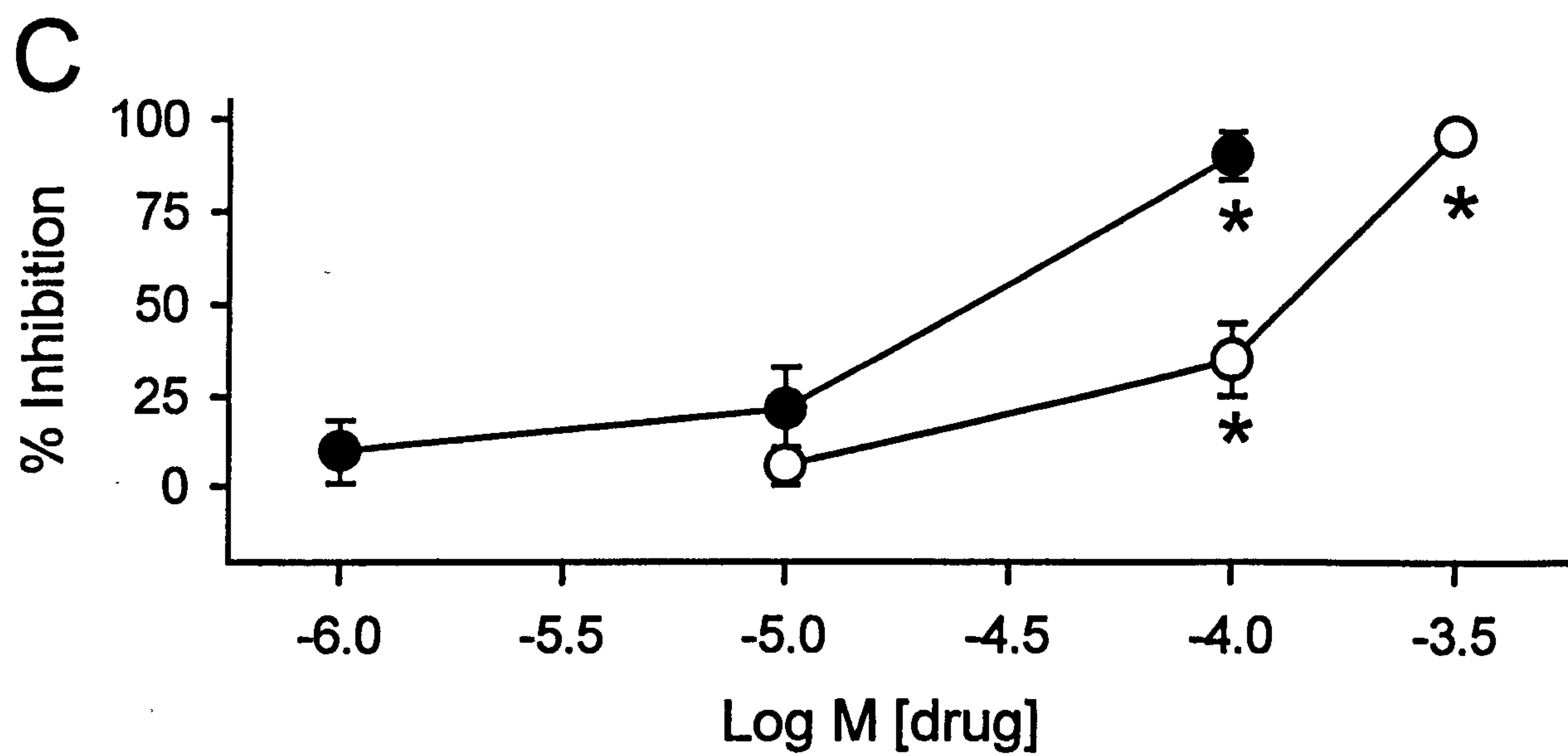
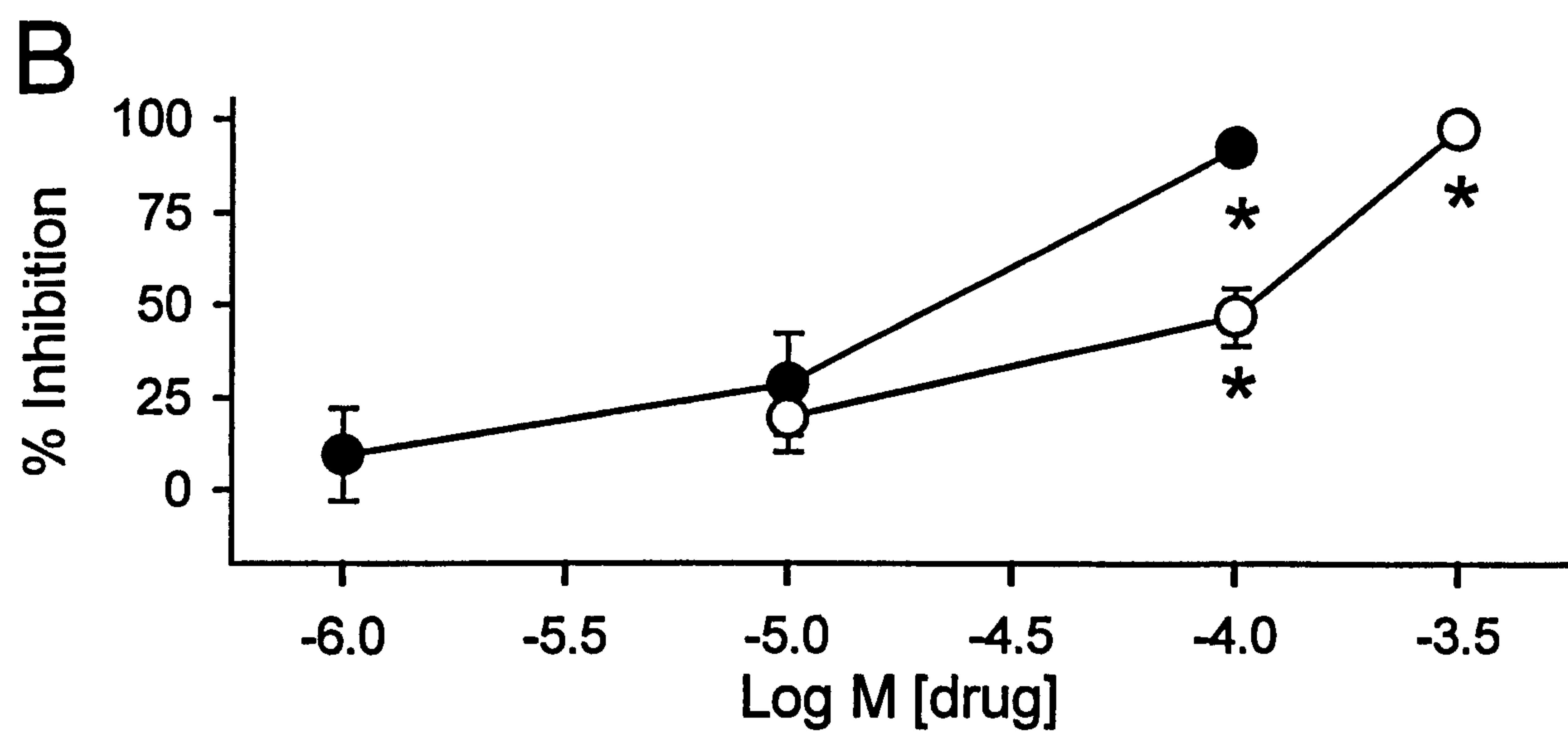
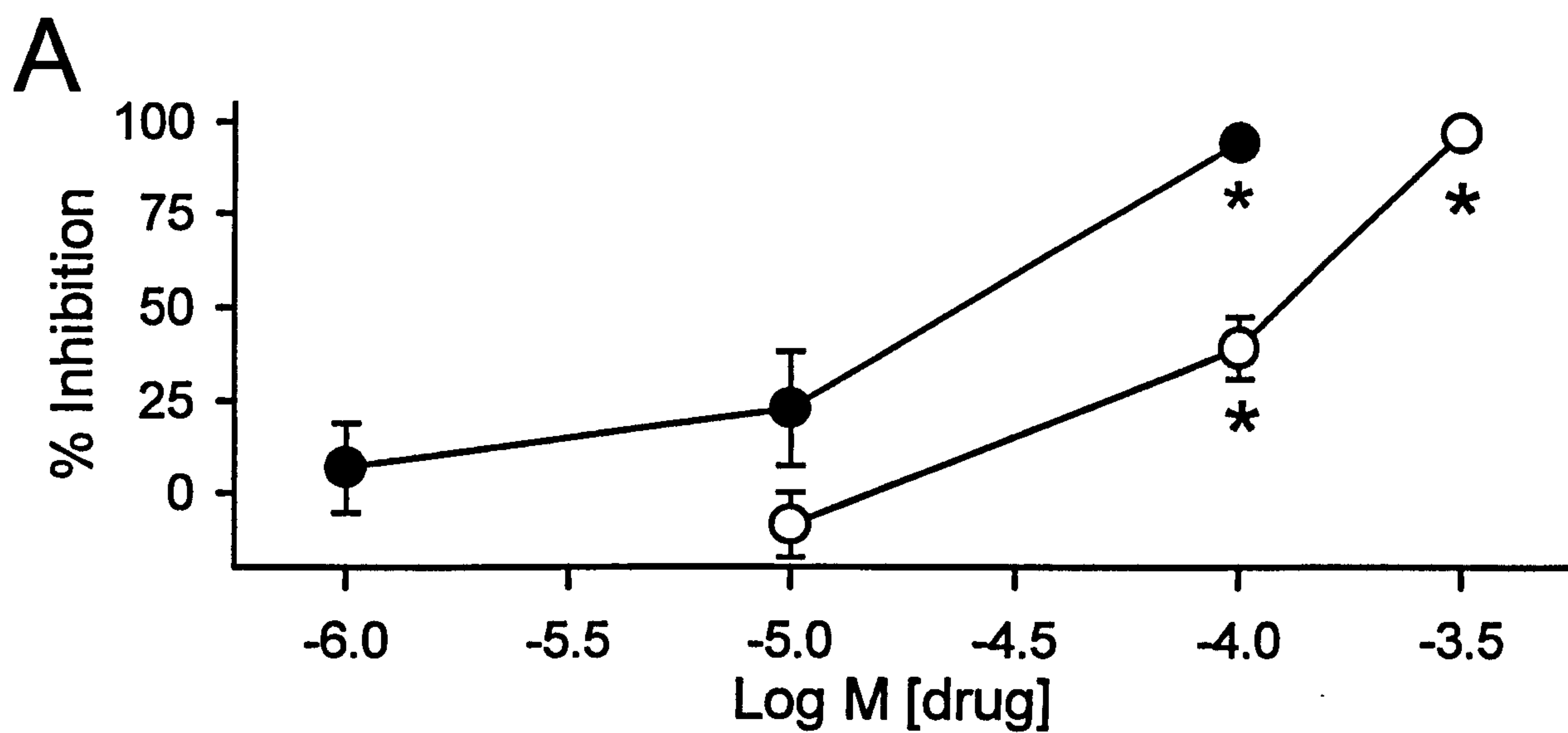
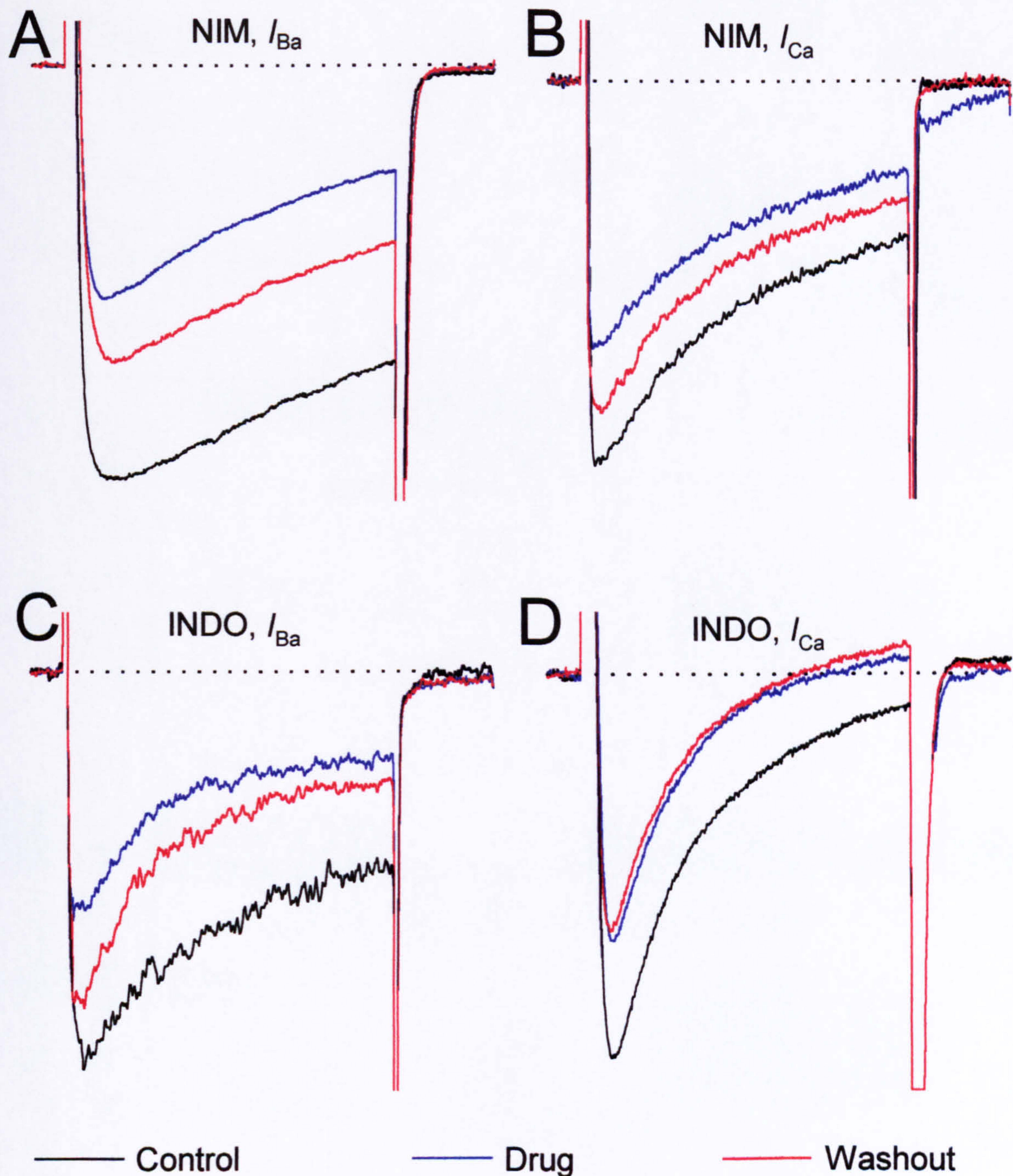
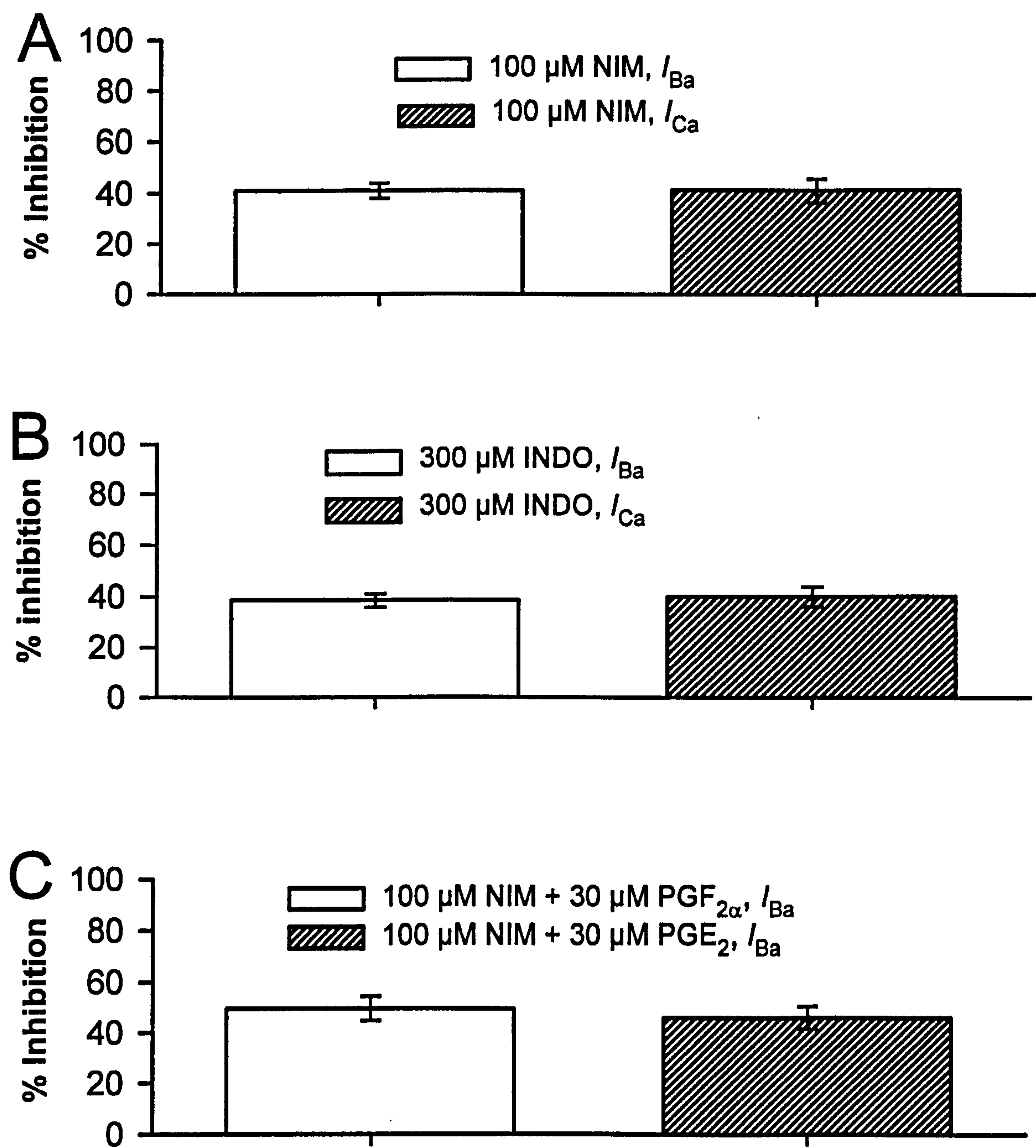


Fig 4.3. Block of I_{Ba} and I_{Ca} by nimesulide and indomethacin



Examples of currents elicited by 200 ms step depolarisations to +10 mV from V_H -50 mV, showing the effect of 100 μ M nimesulide (NIM) on I_{Ba} (A) and I_{Ca} (B) and the effect of 300 μ M indomethacin (INDO) on I_{Ba} (C) and I_{Ca} (D). Black, blue and red traces correspond to control current, current in the presence of drug and current after washout, respectively. Dotted lines represent zero current.

Fig. 4.4. Inhibition of I_{Ba} and I_{Ca} by NIM and INDO, influence of PGs



Evidence that block of calcium channel current (mean \pm S.E.M. of integrated current) by 100 μ M nimesulide (NIM) (A) or 300 μ M indomethacin (INDO) (B) is not affected by the charge carrier (10 mM Ba^{2+} (I_{Ba}), $n = 11$ for NIM & $n = 5$ for INDO or 1.5 mM Ca^{2+} (I_{Ca}), $n = 7$ for NIM & $n = 6$ for INDO). C: Block of neither I_{Ba} nor I_{Ca} was affected by preincubation with either 30 μ M $PGF_{2\alpha}$ ($n = 6$) or 30 μ M PGE_2 ($n = 6$) (not significantly different from block by nimesulide in the absence of prostaglandin, panel A).

4.1.3. VOLTAGE-DEPENDENCE OF CURRENT BLOCKADE BY NIMESULIDE.

In these experiments a concentration of nimesulide (100 μM) that causes 40-50 % inhibition of Ca^{2+} current was used. Fig 4.5. illustrates the effects of 100 μM nimesulide on the current-voltage curves in six cells resulting when V_H was either -80 (3A and 3C) or -50 mV (3B and 3D). Currents were measured at both peak (3A and 3B) and end of pulse (3C and 3D) (300 ms). Regardless of the holding potential, both peak and end-of-pulse current amplitudes (ie both T- and L- components) were diminished by nimesulide over the whole range of potentials. The apparent thresholds of activation and potentials of maximum current amplitude at either holding potential, however, were unaffected by nimesulide. A complete dose response for the L-component of I_{Ba} at +10 mV is presented in Figure 4.11.

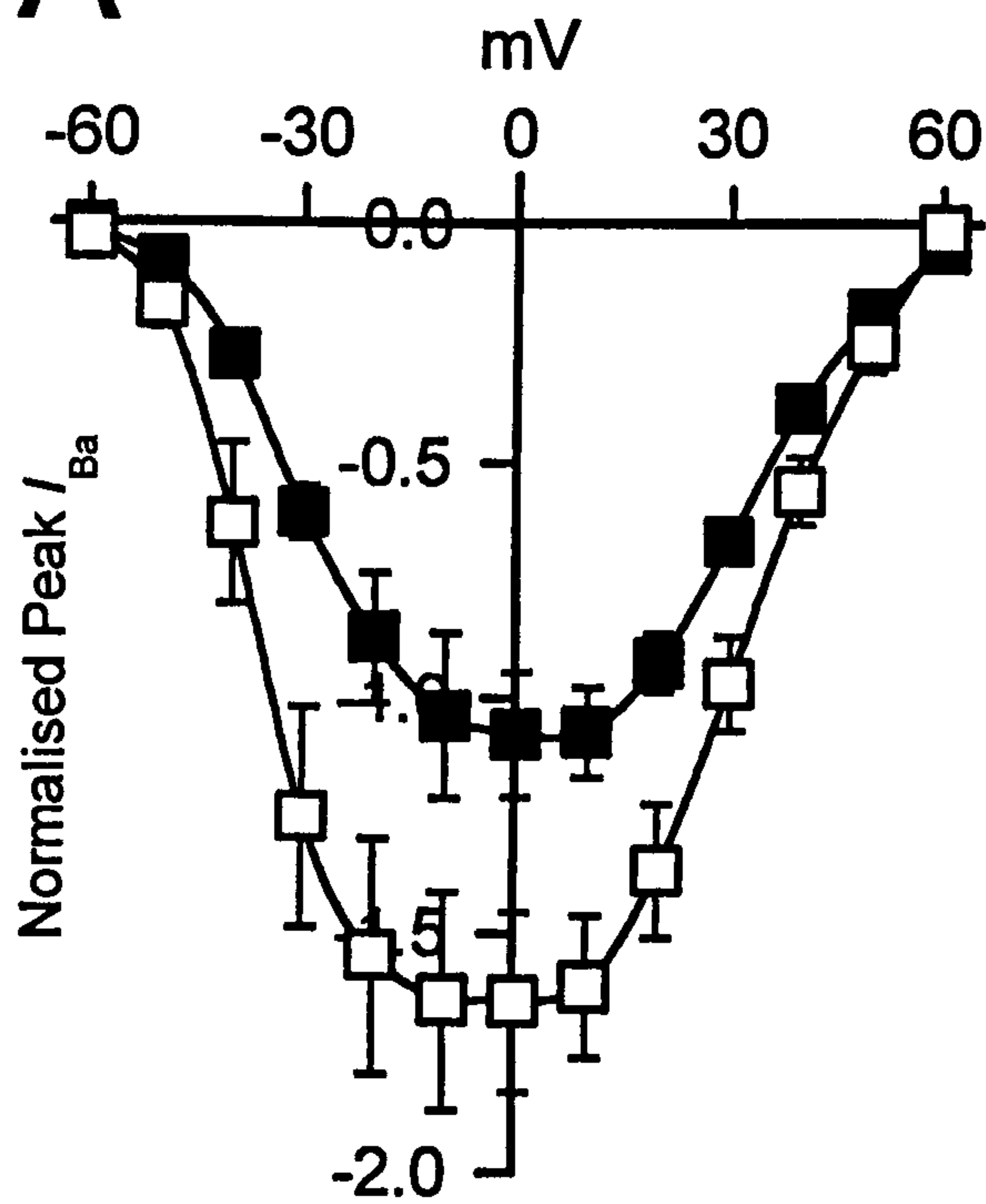
The effect of nimesulide on the availability of the T- and L- components was assessed using the protocol described for Fig 3.3, wherein T- and L- components were isolated by altering holding and test potentials: For the availability of T-component, peak current at -20 mV was measured, and for the L-component, the current was measured at the end of a test pulse to +20 mV. 100 μM nimesulide produced no significant change in $V_{0.5}$ for either L-component (-16.5 ± 1.7 mV in 100 μM NIM vs. -16.4 ± 7.6 mV in PSS, $n=8$) or T-component (-66.6 ± 1.7 mV in 100 μM NIM vs. -65.3 ± 1.8 mV in PSS, $n=6$), indicating that no shift occurred in the potential-dependency of availability for either type of current (Fig 4.6.).

In order to examine possible effects of more prolonged conditioning potentials on the response to nimesulide, the effect of 100 μM drug on currents elicited by pulses to +10 mV were compared when membrane potential was held for several minutes at -40 and -50 mV. Using measurements of the current integral as an overall estimate of current

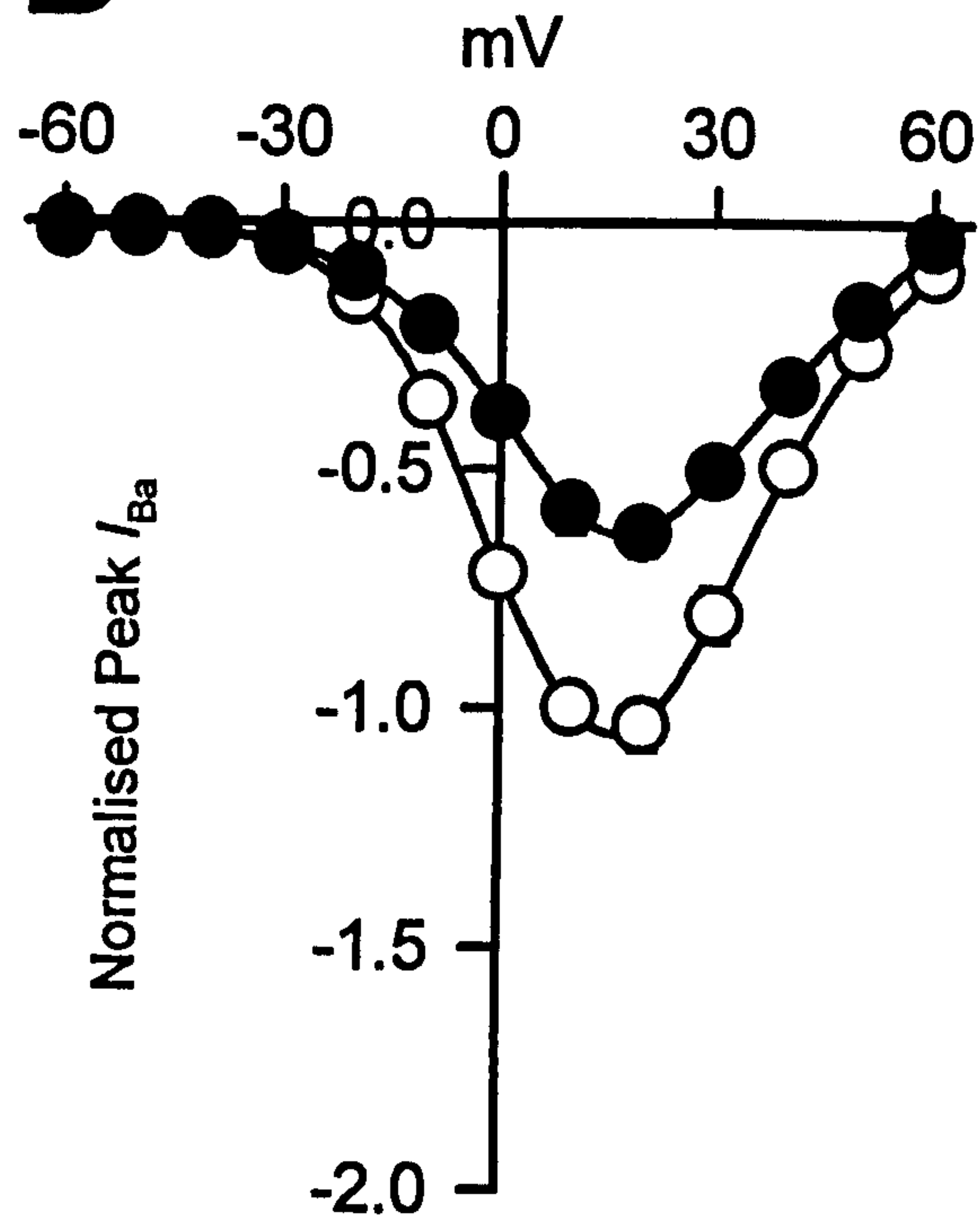
amplitude, there was no significant difference in percent inhibition (resting block) of L-component at the two holding potentials (Fig 4.7A).

FIG. 4.5. VOLTAGE-DEPENDENCE OF BLOCK OF I_{Ba} BY NIMESULIDE

Influence of 100 μ M nimesulide on current/voltage relationships in HMSMCs. **A**, **B**: Peak current from holding potentials (V_H) of -80 mV (**A**) and -50 mV (**B**) in PSS and 100 μ M nimesulide (NIM), with data normalised to the current at +10 mV from V_H -50 mV. **C**, **D**: End of pulse current from V_H -80 mV (**C**) and -50 mV (**D**) in PSS and 100 μ M nimesulide (NIM), with data normalised to the current at +10 mV from V_H -50 mV. All data points are the mean \pm S.E.M. of normalised currents from the same six cells.

A

—□— V_H -80 mV, PSS
 —■— V_H -80 mV, NIM

B

—○— V_H -50 mV, PSS
 —●— V_H -50 mV, NIM

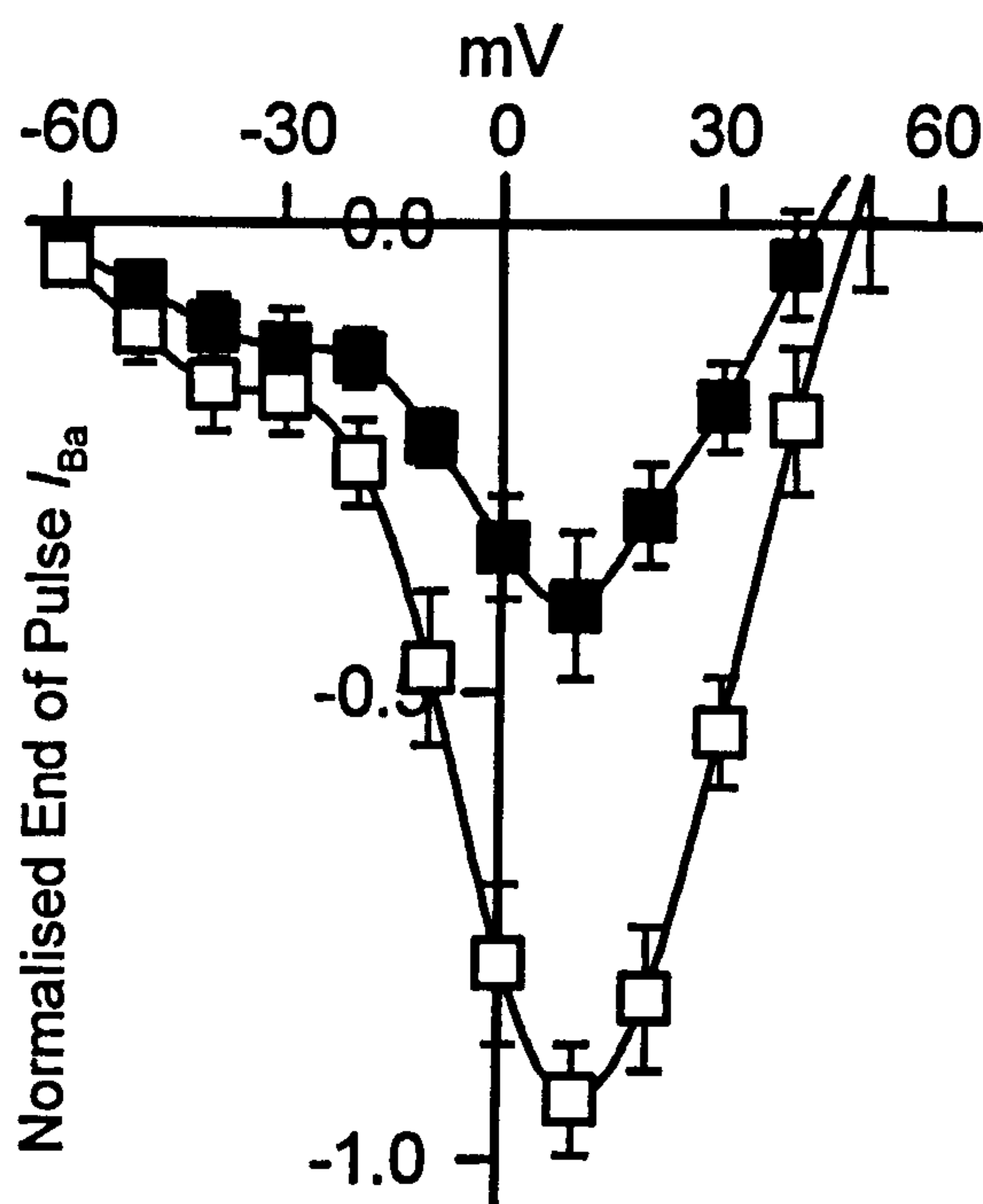
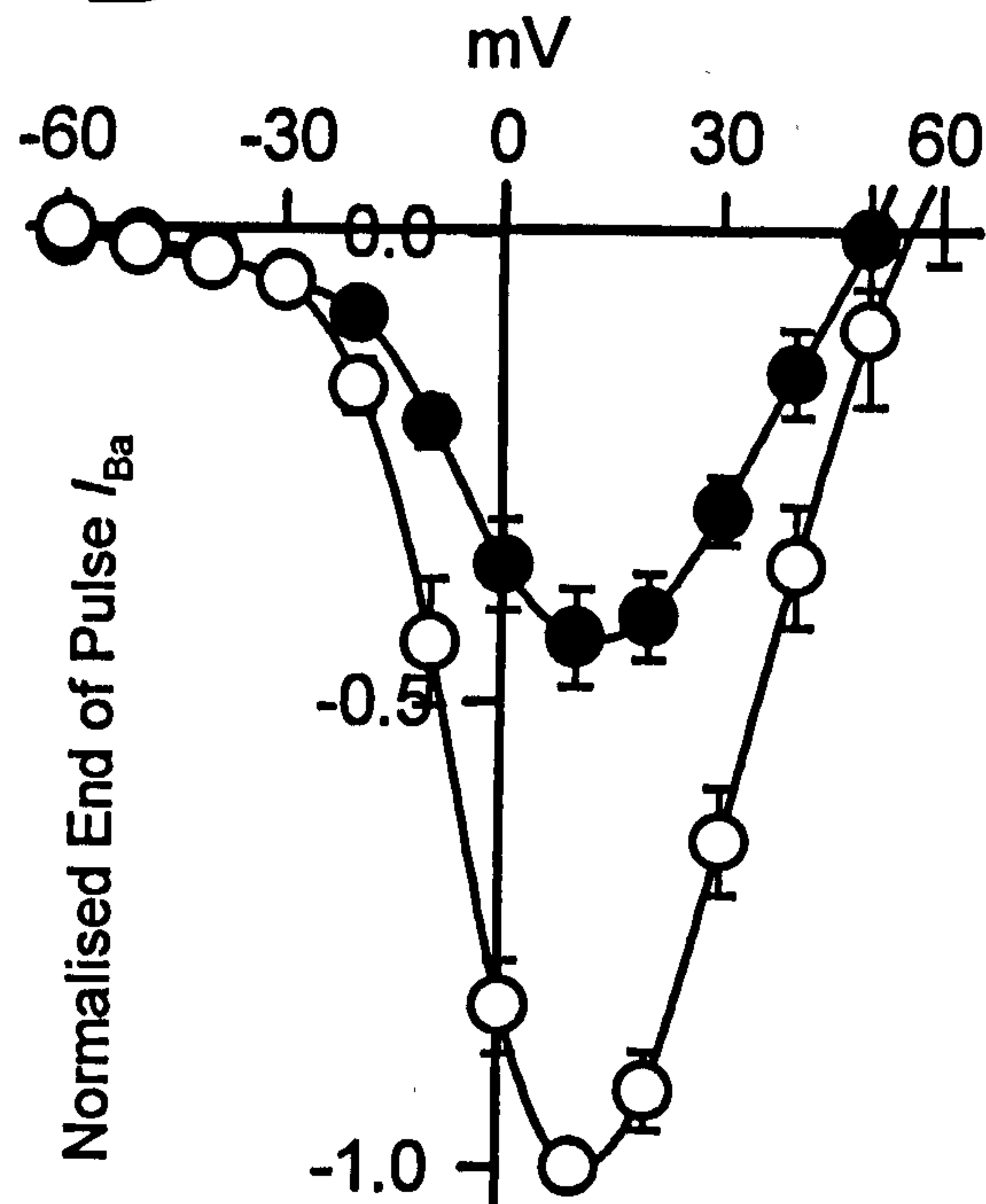
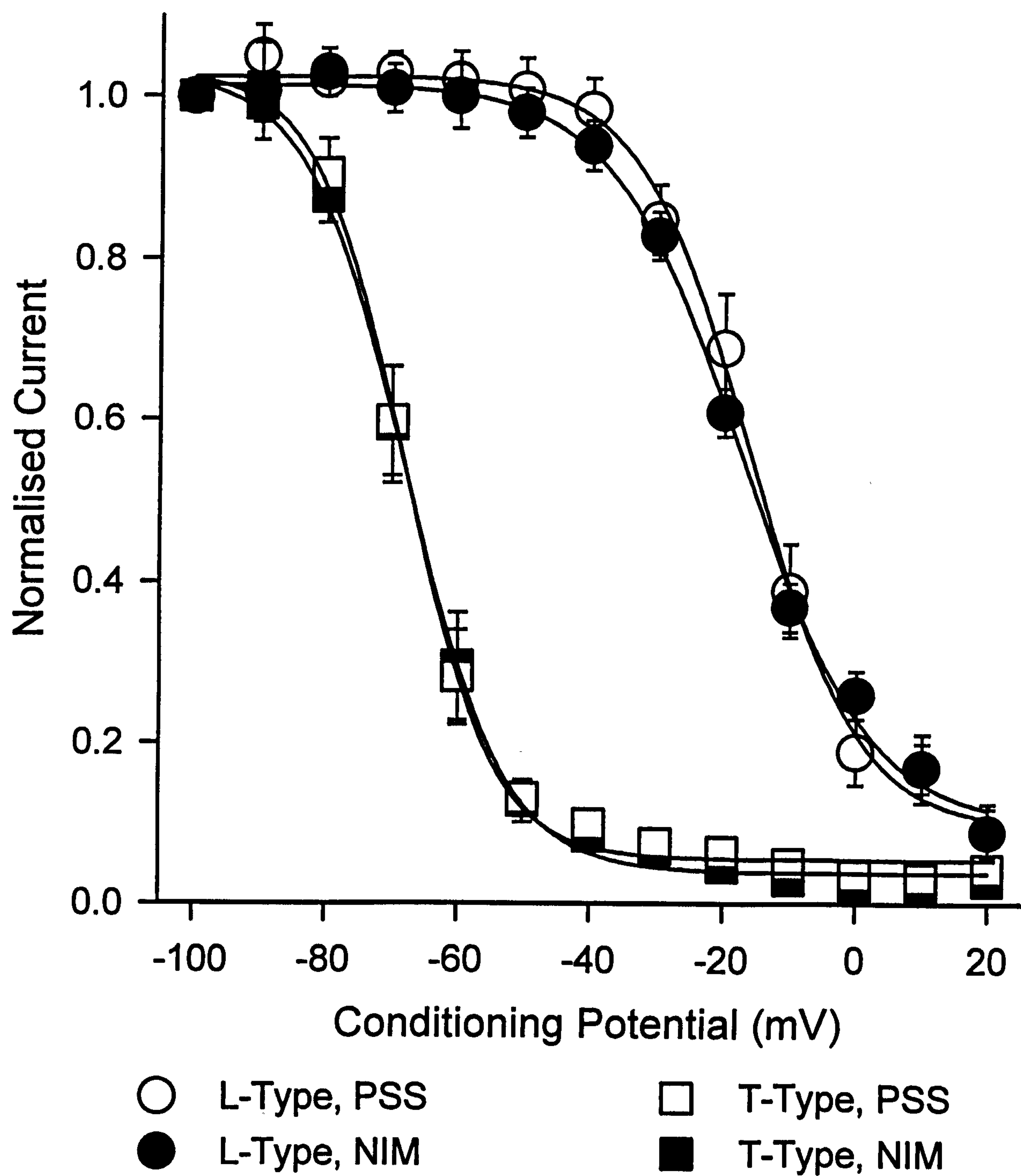
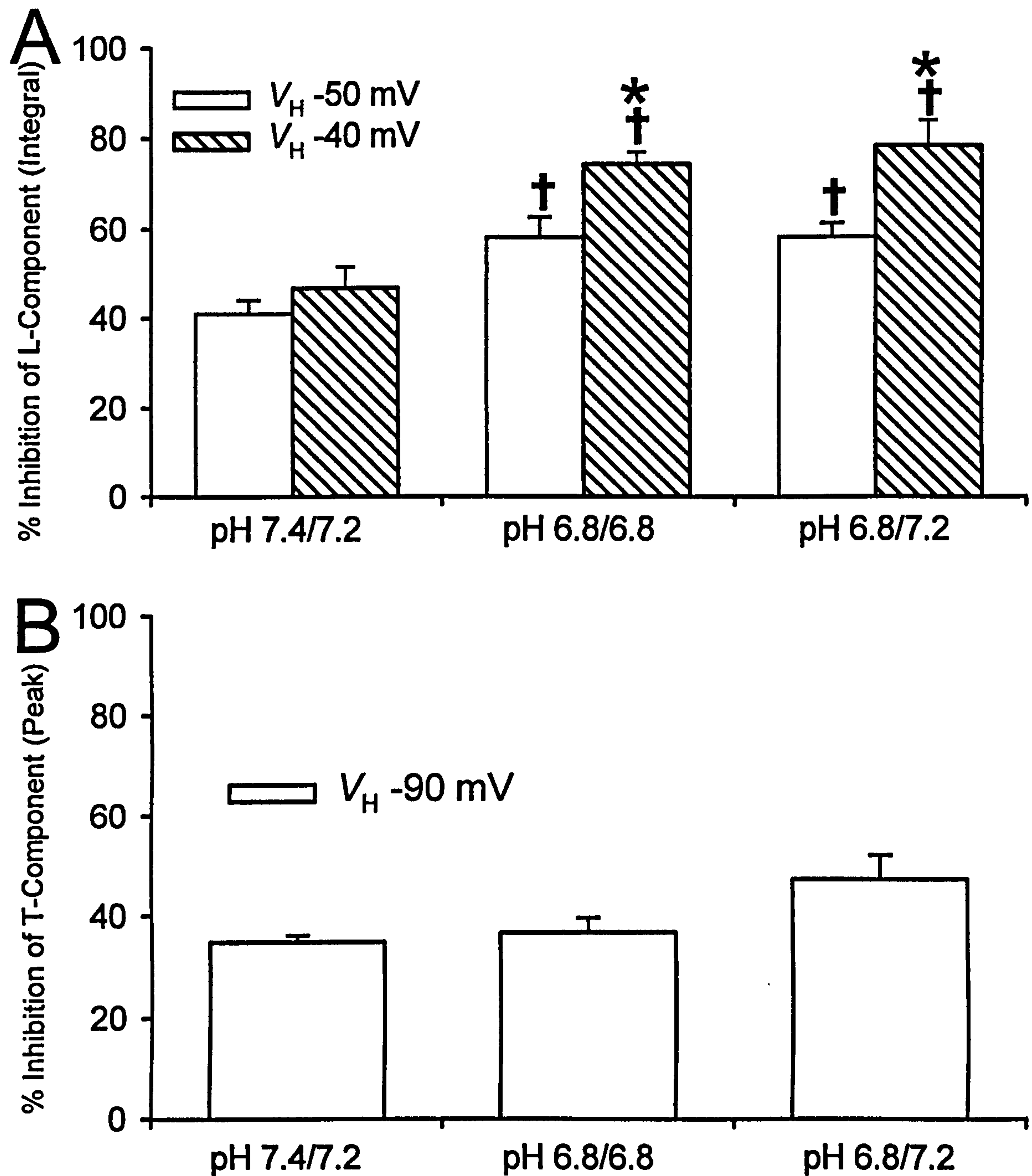
C**D**

Fig 4.6. Effect of nimesulide on I_{Ba} availabilities



Influence of nimesulide on availability of T-type and L-type Ca^{2+} currents in HMSMCs. 5 s pre-conditioning pulses to between -100 mV and +20 mV followed by 300 ms depolarizations to either -20 mV (T-type current, $n = 6$, peak current measured) or to +20 mV (L-type current, $n = 8$, end of pulse current measured) in the absence (PSS) and presence (NIM) of 100 μ M nimesulide.

Fig 4.7. Effect of holding potential and pH on block of I_{Ba} by nimesulide



Summary of the percent inhibition of L-type (A: integrated current) and T-type (B: Peak current) components of I_{Ba} in conditions of differing pH_o/pH_i and different holding potentials. *Inhibition of L-type current was significantly greater from V_H -40 mV than from V_H -50 mV ($P < 0.01$, $n = 6-12$ cells). [†]Inhibition of L-type current was significantly greater at pH 6.8/6.8 and pH 6.8/7.2 than at pH 7.4/7.2 for both V_H -50 mV ($P < 0.05$ by ANOVA) and V_H -40 mV ($P < 0.001$ by ANOVA).

4.1.4. EFFECT OF NIMESULIDE ON Ca^{2+} CURRENT KINETICS

Fig 4.5. illustrates that nimesulide apparently caused a greater inhibition of L-type current at the end of the pulse than at the peak of the current, suggesting that it may have enhanced the apparent rate of inactivation. Currents elicited by depolarizations to +10 mV from V_H -50 mV, in the presence and absence of nimesulide, were therefore fitted with a single component exponential function, as in chapter 3 (equ. 3.2.) and the time constant τ was calculated. Since replacement of Ca^{2+} with Ba^{2+} changes current kinetics, these experiments were also repeated with 1.5 mM Ca^{2+} as the charge carrier. For I_{Ba} , τ was significantly reduced by 100 μM nimesulide (NIM) (PSS, 211 ± 29 ms vs. NIM, 105 ± 8 ms, $P < 0.01$, paired test, a 41 ± 6 % change, $n = 12$), and also for I_{Ca} (PSS, 116 ± 23 ms vs. NIM, 69 ± 6 ms, $P < 0.05$, paired test, a 31 ± 6 % change, $n = 9$). The kinetics of T-type currents were unaffected by nimesulide (PSS, 37 ± 4 ms vs. NIM 33 ± 3 ms, $n = 7$). Unlike nimesulide, indomethacin had no consistent effect on the rate of decay of I_{Ba} (data not shown).

4.1.5. USE-DEPENDENCY AND RECOVERY FROM INACTIVATION OF L-COMPONENT I_{Ba}

The above described enhanced current decay can be explained by binding of nimesulide to either the inactivated state or open state of the channel. Since availabilities were not affected by nimesulide (see above), the possibility of a use-dependent effect of nimesulide on I_{Ba} was examined using the following protocol: A control burst of short (50 ms), high frequency (4 Hz) steps to +10 mV from a -50 mV holding potential, 300 μM nimesulide was applied during a 3 min resting period at -50 mV. This was immediately followed by an identical burst of short rapid steps to +10 mV in the continued presence of nimesulide.

Under control conditions, current amplitude fell progressively during the rapid short depolarizations, but recovered during the three minute rest (Fig 4.8A), and then fell in a

similar manner during a second train of pulses. When nimesulide was added during the rest period, current amplitude was immediately decreased during the first of the subsequent train of test pulses (Fig 4.8B), and then fell further during the train. Results of a number of experiments of this type are shown in Fig 4.8C. Normalization of the data revealed that the fall in current amplitude during the pulse train applied in the presence of nimesulide was exaggerated compared to that observed during the control period (Fig 4.8D).

These results suggested that the effect of nimesulide on the current was use-dependent, possibly because nimesulide unbinding from the channel was incomplete during high-frequency stimulation. In order to investigate this more directly, the effect of nimesulide (300 μ M) on recovery of the L-type current from inactivation was also investigated. Cells were stepped for 500 ms to +10 mV from a holding potential of -50 mV in the presence or absence of nimesulide, and then a second depolarization to +10 mV was imposed, with the interval between the two pulses increased incrementally to determine the time-dependency of recovery. Figure 4.9. shows that the recovery of these currents from inactivation was significantly slowed in the presence of 300 μ M nimesulide.

4.1.6. EFFECT OF LOWERING pH ON BLOCK OF Ca^{2+} CHANNEL CURRENTS BY NIMESULIDE

Nimesulide is a weak acid with a dissociation constant (pK_a) of 6.5 (Magni, 1991). According to the Henderson-Hassalbach equation:

$$\text{pK}_a = \text{pH} + \log_{10} [\text{AH}]/[\text{A}^-], \quad \text{Equ. 4.1.,}$$

where [AH] is the un-ionised drug concentration and $[\text{A}^-]$ is the concentration of the ionised form of the drug, nimesulide will be 88.8% ionized at pH 7.4 (pH outside the cell or pH_o) and 83.4% ionized at pH 7.2 (pH inside the pipette and cell or pH_i), and will

become less ionized as the pH is decreased. The modulated receptor hypothesis (see discussion) states that Na^+ and Ca^{2+} channel antagonists in their ionized forms bind preferentially to the open state of the channel, and in their neutral forms interact predominantly with the inactivated and resting states. Whether the degree of resting block by nimesulide and the effect of nimesulide on channel inactivation were altered when both pH_o and pH_i were lowered to 6.8 (*ie* $\text{pH}_o/\text{pH}_i = 6.8/6.8$) (66.6% ionization; a trebling of the neutral drug concentration) were therefore investigated.

In addition, in order to evaluate whether the ionized form of the drug played any role in channel blockade, the effect of 100 μM nimesulide on resting block and steady-state inactivation was also evaluated in cells where the pH_o was 6.8 and pH_i was 7.2 (*ie* $\text{pH}_o/\text{pH}_i = 6.8/7.2$). Due to ion trapping, these conditions result in a similar steady-state intracellular concentration of neutral drug as with pH 6.8 on both sides of the membrane, but increase the steady-state intracellular concentration of ionized drug by 2.5 fold.

Estimates of the degree of resting block of the T- and L- components of I_{Ba} produced by 100 μM nimesulide at the pH_o/pH_i of 7.4/7.2, 6.8/6.8 and 6.8/7.2 are described in Fig 4.7. The T-component was isolated using 300 ms test depolarizations to -20 mV from V_H -90 mV. Using this protocol, and due to the presence of the large T-current in these cells, peak current was approximately 90 % T-component. The L-component of I_{Ba} was isolated by applying 300 ms steps to either +10 or +20 mV from V_H -50 mV and measuring the integral of the current (at V_H -50 mV, T-current is more than 90 % inactivated). Percent inhibition of the L-component but not the T-component of I_{Ba} by 100 μM nimesulide was significantly greater under both sets of lower pH conditions than at 7.4/7.2 (L-component: $P < 0.05$ by ANOVA, $n = 9-12$ cells; T-component: $P = \text{NS}$ by ANOVA, $n = 6-7$ cells).

As shown in Fig 4.10., inactivation curves when $\text{pH}_o/\text{pH}_i = 6.8/6.8$ for L-component (isolated using a +20 mV test pulse and measuring current at the end of the pulse) and T-

component (isolated using a -20 mV test pulse and measuring peak current) were both significantly shifted to the left by 100 μ M nimesulide, giving a mean shift in the $V_{0.5}$ of -7.3 ± 1.3 mV for L-component (from -17.7 ± 1.0 mV in PSS to -25.0 ± 1.5 mV in NIM, $n=6$, $P < 0.01$, paired t-test) and -4.6 ± 0.8 mV for T-component (from -63.4 ± 2.1 mV in PSS to -68.0 ± 2.7 mV in NIM, $n=6$, $P < 0.01$, paired t-test). Changing the pH *per se* did not significantly affect the availability of either L-component ($V_{0.5}$ -16.5 ± 1.7 mV at $\text{pH}_o/\text{pH}_i = 7.4/7.2$) or T-component ($V_{0.5}$ -66.6 ± 1.7 mV at $\text{pH}_o/\text{pH}_i = 7.4/7.2$).

Nimesulide also produced a similar hyperpolarising shift in the inactivation of both L- and T-components of I_{Ba} when $\text{pH}_o/\text{pH}_i = 6.8/7.2$. The mean shift in $V_{0.5}$ was -9.6 ± 2.2 mV for L-component (from -16.2 ± 2.1 mV in PSS to -25.8 ± 1.1 mV in nimesulide, $n=6$, $P < 0.01$, paired t-test) and -5.9 ± 0.9 mV for T-component (from -61.7 ± 3.3 mV in PSS to -67.6 ± 4.1 mV in nimesulide, $n=7$, $P < 0.01$, paired t-test).

To confirm this apparent voltage-dependence of block at the lower pH_o/pH_i (either 6.8/6.8 or 6.8/7.2) the apparent inhibition of the L-component of I_{Ba} by 100 μ M nimesulide was further estimated using a more depolarized V_H of -40 mV. This blockade was significantly greater than from V_H -50 mV, both at $\text{pH}_o/\text{pH}_i = 6.8/6.8$ and 6.8/7.2 ($P < 0.05$) but not at $\text{pH}_o/\text{pH}_i = 7.4/7.2$ (Fig 4.7.). From V_H -40 mV, there was a similar significant variation in the degree of inhibition between the three pH conditions from V_H -50 mV ($P < 0.001$ by ANOVA, $n=6-9$ cells).

A full dose-response for inhibition of L-component by nimesulide at $\text{pH}_o/\text{pH}_i = 7.4/7.2$ is presented in Fig 4.11. (squares). This mean data was fitted to a four parameter logistic function:

$$y = (100-a)/1+((EC_{50}/x)^b) \quad \text{equ. 4.2}$$

where a is the percentage of current not inhibited by nimesulide and b is the Hill coefficient (normally 1 for a single binding site). An EC_{50} of 136 μ M and a Hill coefficient of 0.81 were obtained and a was zero. Further dose-responses were constructed using $pH_o/pH_i = 6.8/6.8$ (circles) or $6.8/7.2$ (triangles) from V_H -40 mV. Sigmoidal curves were also fitted to these two sets of mean data and estimates of EC_{50} values of 35.6 μ M and 36.0 μ M, and Hill coefficients of 1.08 and 1.02, respectively were obtained, and a was also zero in both cases. The similar sensitivity of the current to nimesulide under these two sets of conditions, together with the similar shift in the availability (see above), suggest that the concentration of ionised drug inside the cell (which is 2.5 times greater when $pH_o/pH_i = 6.8/7.2$ than when it is $6.8/6.8$ or $7.4/7.2$) does not influence the degree of inhibition.

As at normal physiological pH (*ie* $pH_o/pH_i = 7.4/7.2$, see above), current decay of the L-component was accelerated by nimesulide when pH_o/pH_i was $6.8/6.8$ (τ : PSS, 142 ± 19 ms vs. 100 μ M NIM, 75 ± 2 ms, $n=9$, $P<0.01$ paired t-test) and when it was $6.8/7.2$ (τ : PSS, 161 ± 21 ms vs. 100 μ M NIM, 90 ± 7 ms, $n=9$, $P<0.01$ paired t-test). The percentage change in τ was similar under all three sets of pH conditions ($7.4/7.2$: 41 ± 6 %, $6.8/6.8$: 41 ± 6 %, $6.8/7.2$: 42 ± 3 %).

FIG 4.8 USE-DEPENDENCE OF I_{Ba} BLOCK BY NIMESULIDE

Currents elicited by a 7 min burst of short and rapid (50 ms, 4 Hz) depolarizations to +10 mV from a holding potential of -50 mV, followed by a 3 min rest period at -50 mV and then a further 7 min burst of short rapid pulses to +10 mV. Nimesulide was applied at the start of the rest period and was present throughout the second burst of rapid pulses. **A:** Representative control experiment showing traces before (PSS1) and after (PSS2) a 3 min rest period, in the absence of nimesulide. **B:** Representative current traces before (PSS) and after (NIM, 300 μ M nimesulide) the 3 min rest period, respectively. Every fourth current trace is shown. **C:** Effect of nimesulide on peak current in 4 cells (mean \pm S.E.M.). **D:** Same data as in **C** normalised to the zero time point, showing % inactivation of current.

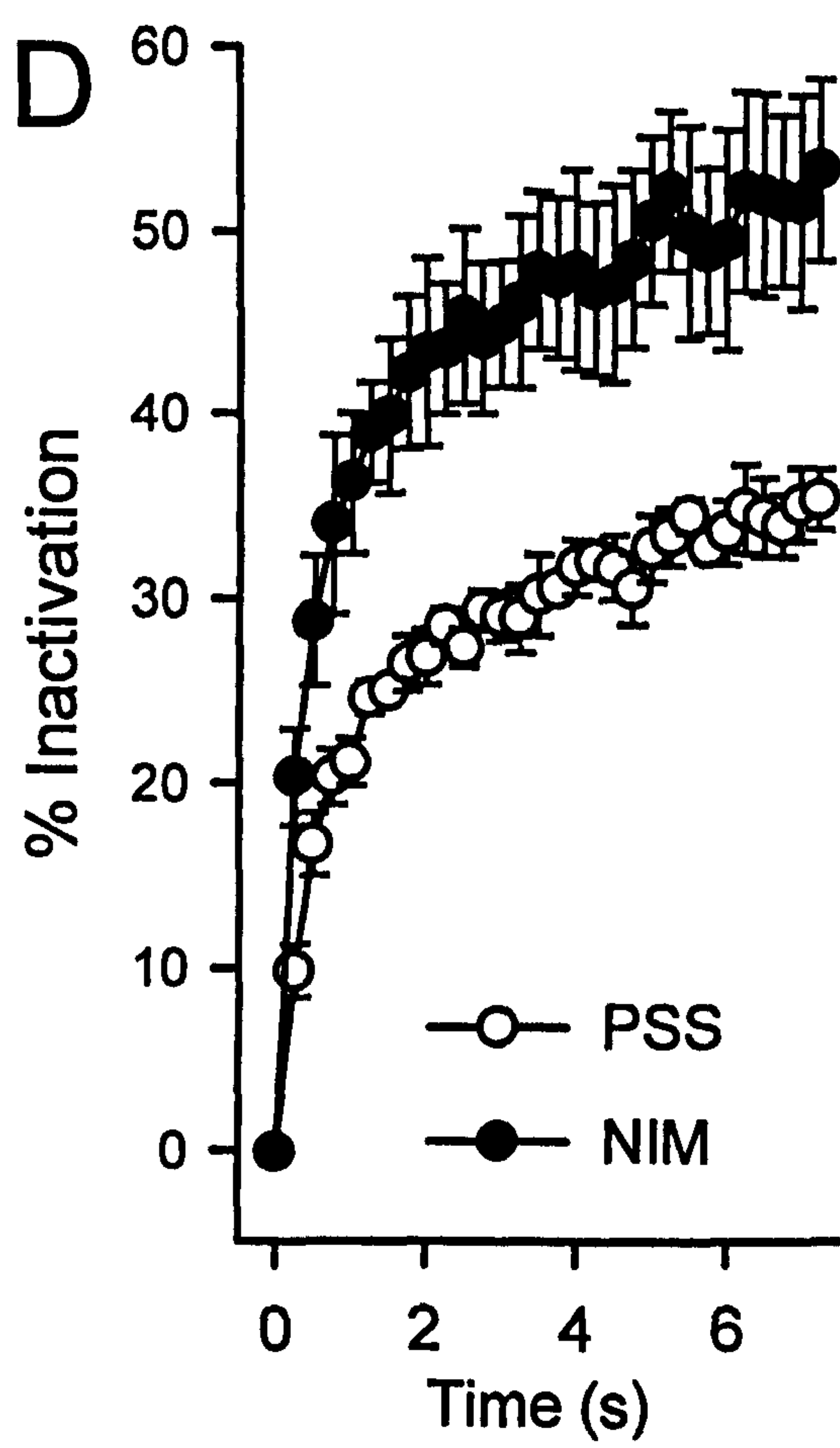
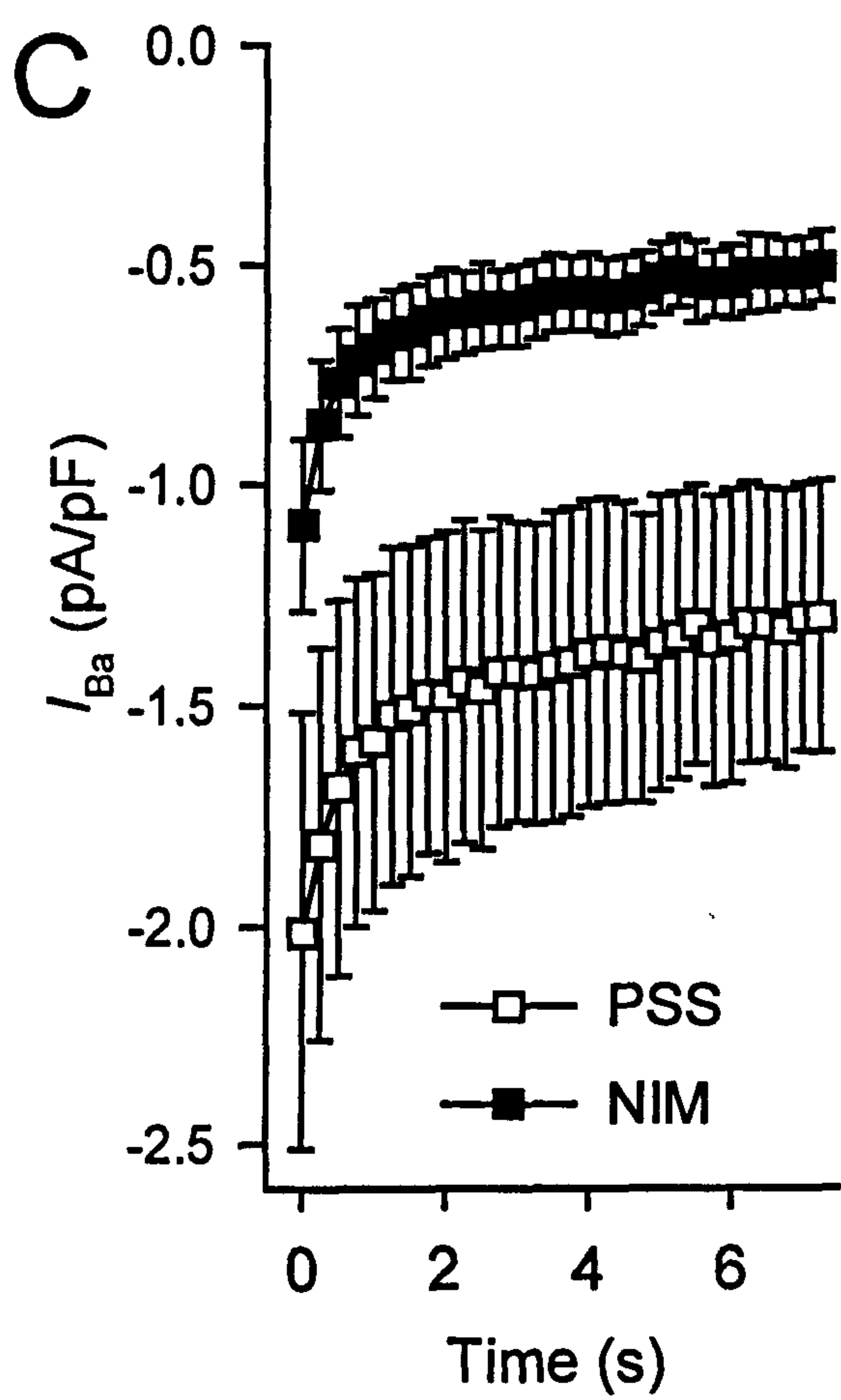
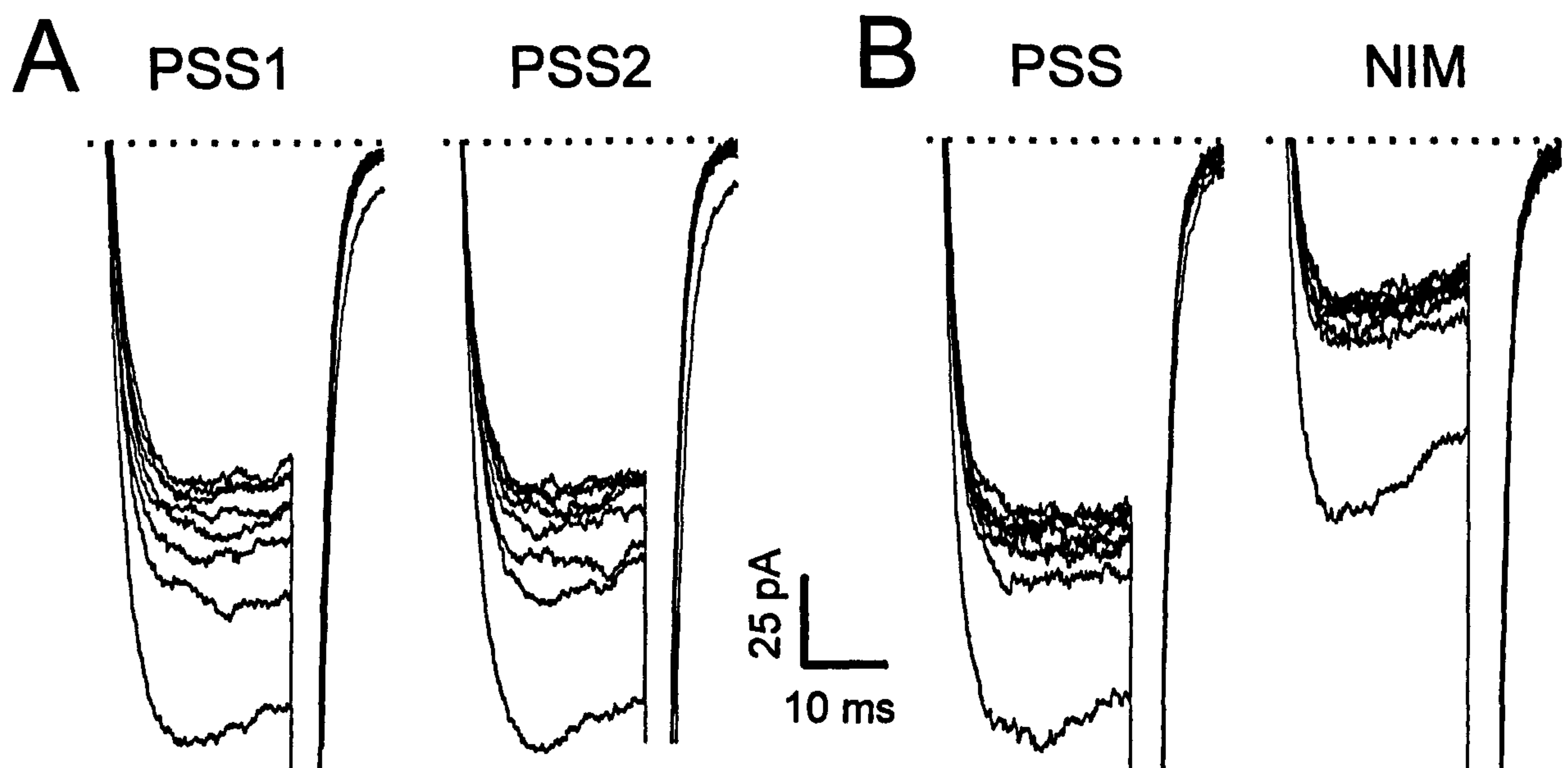


FIG 4.9. EFFECT OF NIMESULIDE ON I_{Ba} RECOVERY FROM INACTIVATION

500 ms depolarization pulses to +10 mV from V_H -50 mV followed at increasing intervals by a further 50 ms pulse to +10 mV. **A & B:** Representative traces showing currents in the presence of PSS (**A**) and 300 μ M nimesulide (NIM) (**B**) in the same cell. **C:** % inactivation at each time interval, in the absence (PSS) and presence of 300 μ M nimesulide (NIM), calculated by taking the peak current amplitude at each 50 ms pulse as a percentage of the corresponding 500 ms pre-pulse (mean \pm S.E.M., $n=5$ cells). * $P < 0.05$. The points are joined by spline curves.

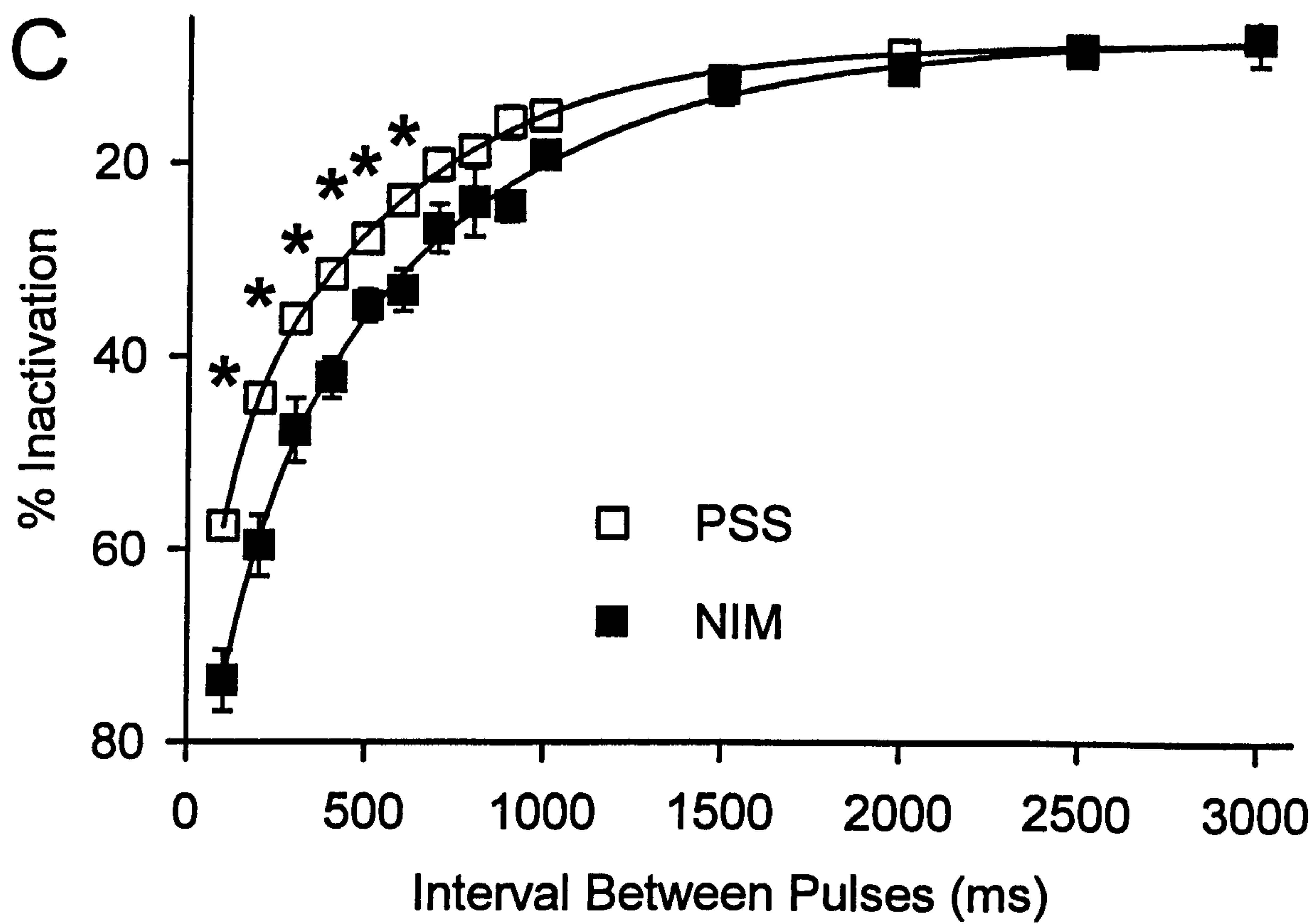
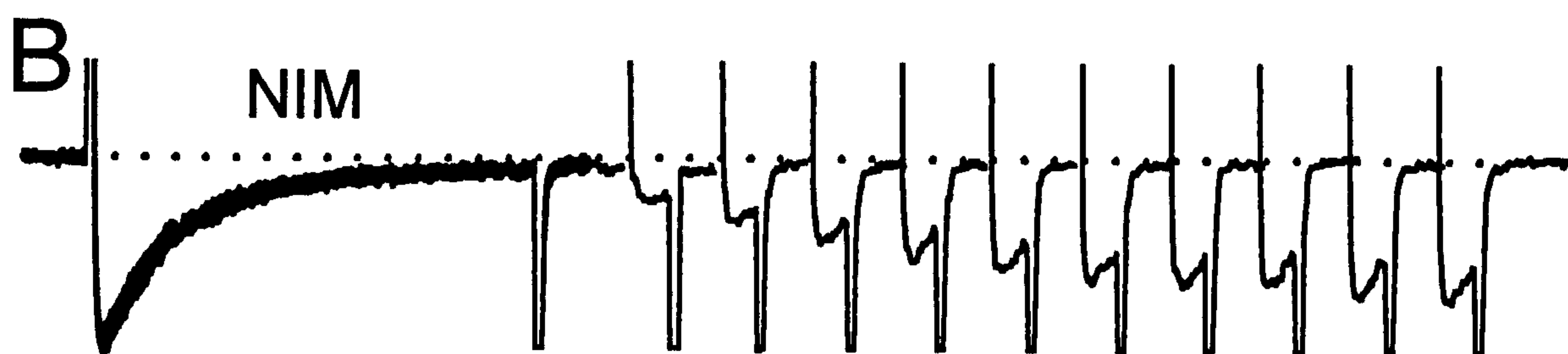
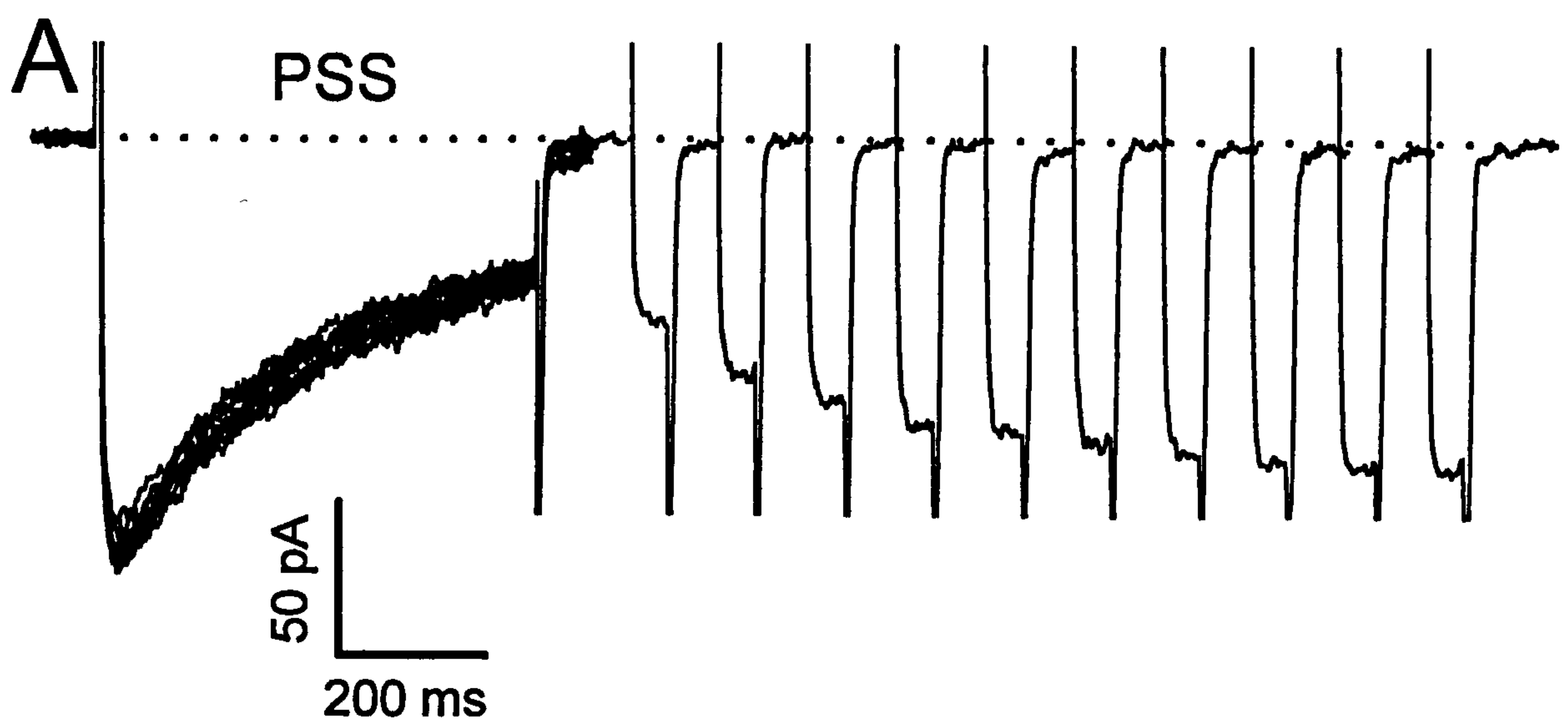
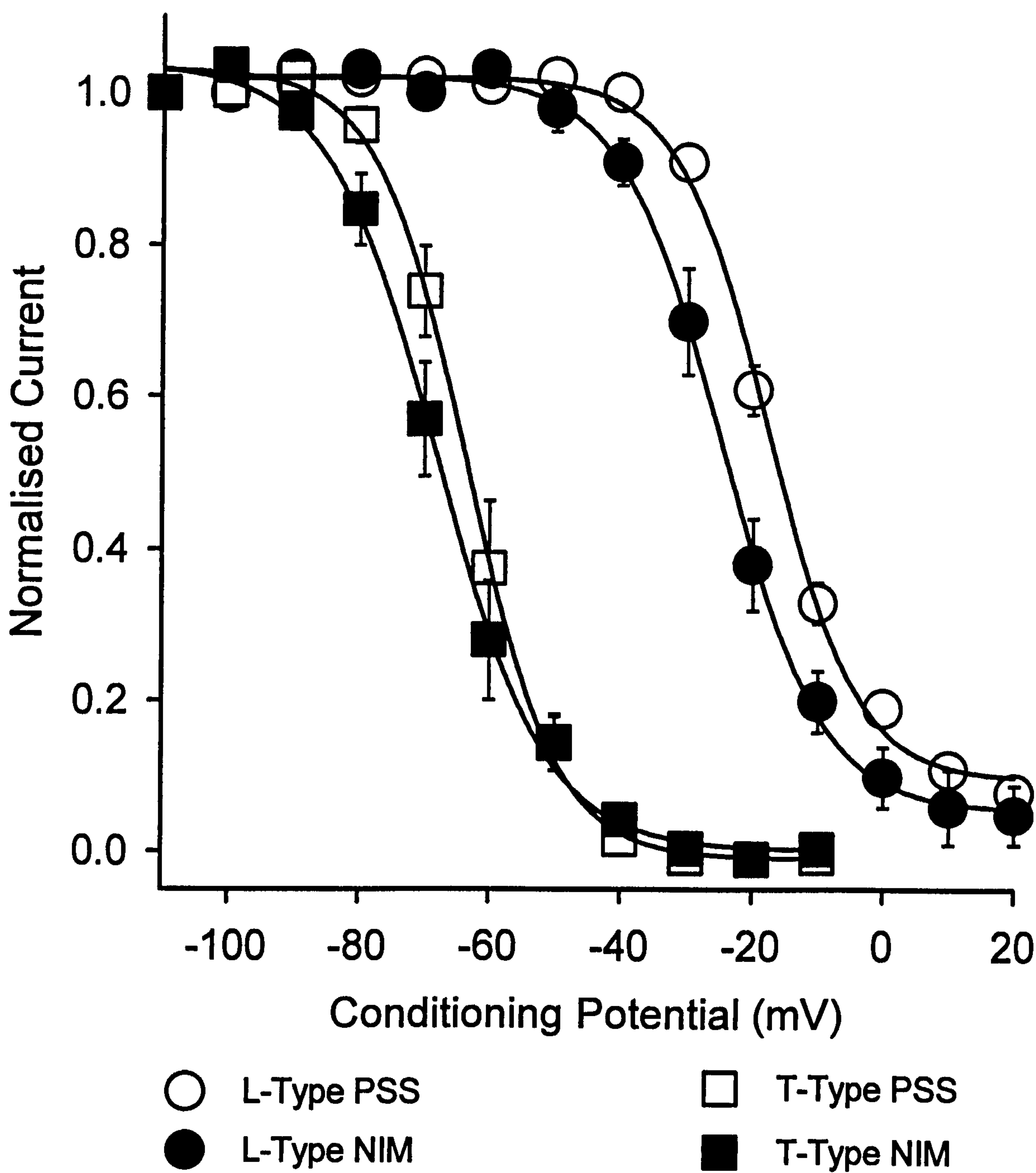
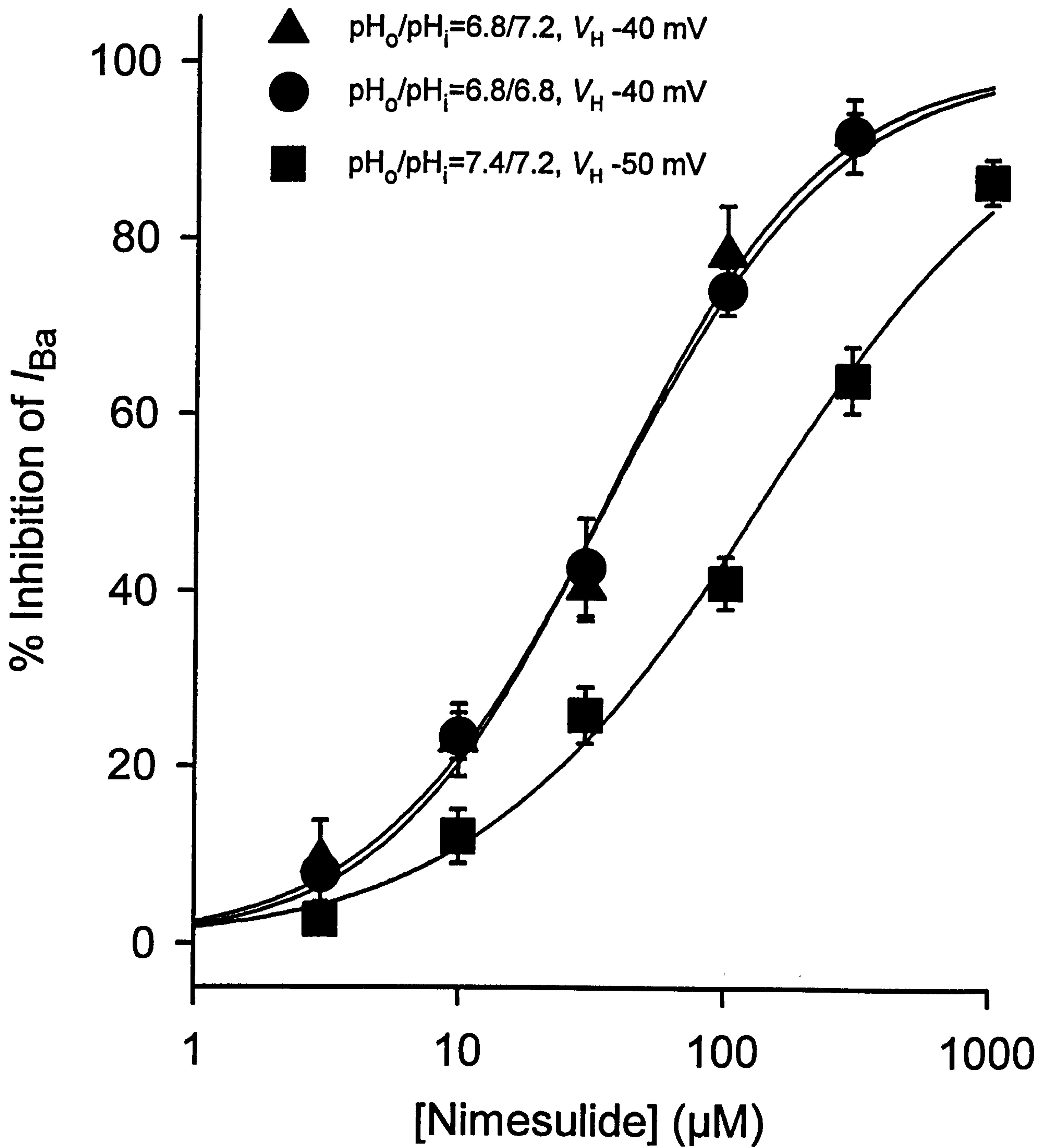


Fig 4.10. Effect of nimesulide on I_{Ba} availabilities at lower pH



Influence of nimesulide on availability of T- and L-components of I_{Ba} in HMSMCs when pH_o/pH_i is 6.8/6.8. 5 s pre-conditioning pulses to between -110 mV and +20 mV followed by 300 ms depolarizations to either -20 mV (T-component, $n = 6$, peak current measured) or to +20 mV (L-component, $n=6$, end of pulse current measured) in the absence (PSS) and presence (NIM) of 100 μ M nimesulide.

Fig 4.11. Concentration-dependence of I_{Ba} block by nimesulide



Effect of extracellular (pH_o) and intracellular pH (pH_i) on the concentration-dependent inhibition of L-type Ca^{2+} current by nimesulide. Points represent the mean \pm S.E.M. of integrated current at +10 mV in 9-12 cells at each concentration. Steady state inhibition at each concentration was reached within 3 min.

4.2. DISCUSSION

4.2.1. NIMESULIDE

Nimesulide (N-(4-nitro-2-phenoxyphenyl)-methane-sulfonilamide), is a non-steroidal anti-inflammatory drug (NSAID) of the sulfonilamide class. It has been used successfully throughout Europe as an anti-inflammatory and analgesic drug and has better gastrointestinal tolerability than other NSAIDs. The anti-inflammatory properties of nimesulide stem from its ability to inhibit prostaglandin production by the cyclooxygenase enzyme (COX), as well as to scavenge leucocyte generated oxygen free radicals and inhibit leucocyte phosphodiesterase type IV (Davis & Brogden, 1994). COX exists in two isoforms: COX-1 which is constitutively expressed where prostaglandin synthesis is constant, and COX-2 which is inducible and associated with inflammation. Within the fetoplacental unit, COX-2 and not COX-1 is expressed in fetal membranes and myometrium and upregulated with labour (Slater *et al.*, 1995, 1997), whereas COX-1 is the predominant isoform in systemic fetal tissues. Nimesulide has a 30-100 fold selectivity for COX-2 (Taniguchi *et al.*, 1995; Miralpeix *et al.*, 1997) and has been shown not to affect prostaglandin synthesis in tissues in which COX-1 is the predominant isoform (Bianco *et al.*, 1991; Tavares *et al.*, 1995). A preliminary clinical report has suggested that it may be a potentially useful tocolytic agent (Sawdy *et al.*, 1997a), enabling the targeting of COX-2 and avoiding the harmful fetal side effects that preclude the use of non-selective COX-inhibitors such as indomethacin (Norton *et al.*, 1993, Table 1.5.).

4.2.2. EFFECTS OF NIMESULIDE AND INDOMETHACIN ON SPONTANEOUS MYOMETRIAL CONTRACTION *IN VITRO*

Both nimesulide and indomethacin suppressed spontaneous contractility of pregnant human myometrium *in vitro*. A number of previous studies have evaluated the effect of indomethacin, with variable results. For example, Vane and Williams (1973) found that 11

μM indomethacin gradually abolished both spontaneous myometrial contractions and uterine prostaglandin production in pregnant rat uterus and Garrioch (1978) observed that 50 μM indomethacin gradually abolished spontaneous contractions in non-pregnant and pregnant human myometrial strips. A similar effect of indomethacin on human myometrial strips was also demonstrated by Johnson *et al.* (1975) but at much higher concentrations (< 50% inhibition with 278 μM indomethacin). Similarly, Crankshaw & Dyal (1994) more recently found that indomethacin up to 100 μM had no effect on spontaneous contractions of pregnant human myometrium *in vitro*. In the present study, indomethacin inhibited spontaneous myometrial activity over a concentration range somewhat lower than that found by Johnson *et al.* (1975), with almost complete inhibition of activity at 300 μM , and nimesulide, the effects of which on *in vitro* myometrial contractility have not previously been evaluated, was approximately three-fold more potent than indomethacin.

From these experiments it was apparent that neither drug was inhibiting contraction at concentrations known to inhibit COX activity. For example, nimesulide and indomethacin reduced PGE_2 production by human fetal membranes by 75 % and 82 % respectively at 1 μM (Sawdy *et al.*, 1997b). Therefore the concentrations of both drugs required to suppress spontaneous *in vitro* contractions of human myometrium were approximately 2 orders of magnitude higher than those needed to inhibit uterine COX. These data agree with several previous reports which show that indomethacin, although more potent against COX-1 (60-fold), also inhibits COX-2 with micromolar (Mitchell *et al.*, 1993) or even submicromolar IC_{50} values (Miralpeix *et al.*, 1997, Yamada *et al.*, 1997). Similarly, nimesulide inhibits COX-2 at micromolar or submicromolar concentrations (Casolaro *et al.*, 1993; Miralpeix *et al.*, 1997).

The fetal membranes (amnion and chorion) and decidua are thought to be the principle sites of synthesis of oxytocic prostaglandins (PGE_2 and $\text{PGF}_{2\alpha}$) within the human uterus

and fetoplacental unit (Skinner & Challis 1985) and, even though COX-2 mRNA is present in pregnant human myometrium (Slater *et al.*, 1995, 1997), the importance of the myometrium itself as a source of oxytocic prostaglandins is uncertain, since the principle prostaglandin product of myometrium is prostacyclin (Bamford *et al.*, 1980), which has no effect upon contractility (Crankshaw & Dyal, 1994). In contrast to early *in vitro* contractility experiments with indomethacin therefore, where it was concluded that intrinsic prostaglandin production was involved in maintenance of the spontaneous myometrial contractions (Vane & Williams, 1973), the observations of the present study suggest that neither indomethacin nor nimesulide were likely to be suppressing *in vitro* contractility *via* an inhibition of prostaglandin production.

As an alternative mode of action then, perhaps the relaxant effect of the two drugs was due to inhibition of myometrial Ca^{2+} channels. In the present study, both nimesulide and indomethacin approximately half-maximally reduced both I_{Ba} and I_{Ca} in human myometrial myocytes at concentrations which completely inhibited contractility. Nimesulide was approximately three-fold more potent as an antagonist of current than indomethacin, apparently mirroring the relative potency of the two drugs as inhibitors of myometrial contractility. Furthermore, the inhibition of the Ca^{2+} channel current was unlikely to be secondary to an effect on prostaglandin production by the isolated cells, since the presence of either PGE_2 or $\text{PGF}_{2\alpha}$ in the solution did not significantly alter the extent of current blockade by nimesulide. Indeed, the addition of prostaglandin itself did not significantly affect current amplitude, suggesting that either prostaglandin receptors were damaged by the enzymatic digestion, or that the prostaglandins were unable to directly influence voltage-gated Ca^{2+} channel activity under the voltage-clamp conditions used. These results are therefore consistent with the possibility that, at least in part, both drugs may act as myometrial relaxants *via* Ca^{2+} channel blockade. For this reason the mechanisms of Ca^{2+} channel blockade by nimesulide, the most potent of the two drugs, were studied in more detail.

4.2.3. RESTING BLOCK OF L- AND T-TYPE COMPONENTS BY NIMESULIDE

The block of I_{Ba} by 100 μ M nimesulide at physiological pH ($pH_o = 7.4$, $pH_i = 7.2$) did not appear to demonstrate any dependency on the holding potential. No shift of the inactivation curve of either L-component or T-component of I_{Ba} was observed when 5 second conditioning pulses were used, and block of the current was similar from V_H -50 and -40 mV. Furthermore, nimesulide did not alter the shape of the I - V curve at either holding potential (-80 mV or -50 mV), either when peak current was measured or when current was measured at the end off the pulse, and there was no discernible shift in the apparent threshold of activation or potential of maximum activation.

An interesting aspect of block by nimesulide is its lack of selectivity for either L-type or T-type currents. As discussed in chapter 3, a role for the T-current in myometrial contractility has not been proved, but its electrophysiological properties suggest that it is possible that it may contribute to slow wave or action potential generation. It is possible therefore that block of T-type channels may contribute to nimesulide's inhibitory effect on myometrial contractility.

4.2.4. STATE-DEPENDENT CHANNEL BLOCK BY NIMESULIDE

The observation that considerable block of current was observed during the first depolarization following a 3 min incubation with nimesulide (Fig 4.8C), indicated that the greatest part of the block developed under these conditions was *via* binding of nimesulide to the channel in its closed (resting) state. However, nimesulide also blocked the L-type current significantly more when measured at the end of the 300 ms pulse than at the current peak, suggesting that it might accelerate current decay. This was confirmed by calculating time constants of current decay in the absence and presence of nimesulide.

This effect was similar and significant either with 10 mM Ba²⁺ or with 1.5 mM Ca²⁺ as charge carrier, and was apparently independent of pH. In addition to accelerating current decay, nimesulide significantly enhanced the decline of the current which developed during a train of brief high frequency depolarizations, and also significantly slowed recovery of the current following a 500 ms voltage step. These enhanced current decay and use-dependent properties, suggest that block of I_{Ba} by nimesulide may also be promoted by channel opening (Yeh *et al.*, 1982; Lee & Tsien, 1983).

The phenylalkylamines, including verapamil and its derivative D600, and the benzothiazepine diltiazem, are predominantly frequency- and use-dependent and appear to bind preferentially to the open state of the channel (Lee & Tsien, 1986; McDonald *et al.*, 1993). Furthermore, the degree of use-dependence of a drug may be predicted by the degree of ionization of the drug at physiological pH. This is evident with D600 (pK_a 8.5, 93% ionized at pH 7.4) and verapamil (pK_a 9.7, 96% ionized at pH 7.3) which are both wholly use-dependent at physiological pH (Sanguinetti & Kass, 1984; Uehara & Hume 1985). Diltiazem is intermediate (pK_a 7.7, 67 % ionized at pH 7.4) so that lowering the pH to 6.5 increases the percent of ionized drug to 95 % and increases the degree of use-dependence (Uehara & Hume, 1985). On the other hand, the dihydropyridines such as nifedipine (pK_a 1.0), nicardipine, nisoldipine and nitrendipine are wholly neutral and cause large hyperpolarizing shifts in the steady-state inactivation, indicative of preferential binding to the inactivated state of the channel and demonstrate little or no use-dependence at physiological pH (Sanguinetti & Kass, 1984; Uehara & Hume, 1985; Bean *et al.*, 1986; Terada *et al.*, 1987).

Nimesulide differs from these antagonists, in that due to its sulfonilamide group it is a weak acid (Magni, 1991). Thus lowering the pH from 7.4 to 6.8 decreases the amount of ionized drug and increases the amount of the neutral form of the drug from 11.2 to 33.4 %. This three-fold increase in concentration of neutral drug paralleled the increased block

by nimesulide when pH_o/pH_i was 6.8/6.8 (Fig 4.7.). Furthermore, if the ionised form of the drug was contributing to resting block, when pH_o/pH_i was 6.8/7.2, where the concentration of ionised drug inside the cell would be increased 2.5 fold by an ion trapping effect, and neutral drug concentration would be the same as at 6.8/6.8, the potency of nimesulide would be even greater. Since this was not the case, it can be concluded that the neutral form of the drug is predominantly responsible for block of the resting channel.

In addition, nimesulide caused a 7.3 mV leftward shift of the inactivation curve of the L-component when the pH was 6.8 both in the bath and pipette solutions. According to Bean *et al.*, (1984), this shift can be used to calculate the affinity of the drug for the inactivated state (K_I), as follows:

$$\Delta V_{0.5} = k \ln[(1 + N/K_R)/(1 + N/K_I)] \quad \text{Eqn 4.3.}$$

Where $\Delta V_{0.5}$ is the shift in the inactivation, k is the inactivation slope factor (mean 6.95 mV), and N is the neutral drug concentration. Assuming that only the neutral form of the drug binds to the resting state of the channel, the affinity for the resting state, K_R , is estimated from an approximate EC_{50} of 136 μ M at pH 7.4 of which 11.2 % is in the neutral form and, assuming that changing the pH does not change the affinity of the drug for the channel, giving a K_R of 15 μ M for the neutral form of the drug. With a $\Delta V_{0.5}$ of -7.3 mV at pH 6.8 (where 33.6 % of the drug is neutral), K_I is estimated as 4.1 μ M for the neutral form of the drug.

The possibility that nimesulide binds with a higher affinity to the inactivated state of the channel is entirely consistent with the observation that, at pH_o 6.8, when the holding potential was set at -40 mV there was significantly more block than when the holding potential was -50 mV. The fact that this reduction in holding potential did not cause

additional block or shift the current availability of the L-component at pH_o 7.4, was most likely because the concentration of neutral drug was very low under this condition.

The absence of a significantly greater hyperpolarising shift in current availability when pH_o/pH_i was 6.8/7.2 compared to when it was 6.8/6.8, suggests that the ionized form of the drug does not contribute to inactivated channel blockade.

Taken together these observations are consistent with the concept that nimesulide blocks the Ca^{2+} channel in resting, inactivated and open states. The block of resting and inactivated channels is largely caused by the neutral form of the drug. The effect of nimesulide on current decay was similar under all three sets of pH conditions and was not apparently influenced by the concentrations and relative proportions of neutral and ionized drug in the bath and in the intracellular medium. However, the underlying rate of current decay was also accelerated at the lower pH_i , making interpretation of these data difficult.

Nimesulide also caused a small but significant leftward shift in the availability of the T-component when pH_o/pH_i was either 6.8/6.8 or 6.8/7.2. The extent to which this effect might contribute to nimesulide's potency as a myometrial relaxant is unclear, given the uncertainty over the role of the T-current in myometrial contractility.

4.2.5. POTENTIAL FOR TOCOLYSIS AND INFLUENCE OF pH

It is clear that at normal physiological pH ($\text{pH}_o/\text{pH}_i = 7.4/7.2$) both nimesulide and indomethacin were less effective in inhibiting the Ca^{2+} channel current than in inhibiting contractility. The smaller effect on the current may imply that Ca^{2+} antagonism is only one of several mechanisms by which these drugs suppress contractions. It may also, however, stem from the methodological differences involved in studying contractility and ion channel currents. For example, the duration of the exposure of single cells to nimesulide or indomethacin was effectively limited by the durability of the cells under voltage clamp

conditions and the possibility of slow run-down of the Ca^{2+} current. Finally, no data exist as to the relationship between Ca^{2+} channel blockade and the inhibition of spontaneous activity in this tissue, and it may be that partial reduction of the current has a disproportionate inhibitory effect on action potential activity and contractions.

Whether or not the effects of these drugs on the Ca^{2+} current bear any relevance to their *in vivo* tocolytic effect is unclear. However, total therapeutic plasma concentrations of indomethacin are approximately 1-2 μM , of which 90% is bound to plasma proteins (Berman *et al.*, 1980). Both total and free plasma concentrations of indomethacin are therefore much lower during therapy than those which inhibited myometrial contractility and the Ca^{2+} current *in vitro*. On the other hand, therapeutic total plasma concentrations of nimesulide are relatively high, ranging between 6 and 32 μM (Davis & Brogden, 1994). Nimesulide is 99% bound in plasma (Bree *et al.*, 1993), indicating that its free plasma concentration range is submicromolar. Nimesulide, however, is lipophilic, and its membrane concentration is therefore likely to be much higher than its free plasma concentration. It may be for this reason that nimesulide reaches a concentration in female reproductive organs which is 33 - 50% of the *total* plasma concentration (Davis & Brogden, 1994). In addition, many of the diverse effects of nimesulide which are thought to contribute to its *in vivo* anti-inflammatory action have relatively high ($> 10 \mu\text{M}$) EC_{50} or IC_{50} values when these effects are assayed *in vitro* (Casolaro *et al.*, 1993; Facino *et al.*, 1993; Ottonello *et al.*, 1995). In particular, it has been proposed that many of these actions are related to its ability to inhibit phosphodiesterase (PDE) type IV, the principle enzyme responsible for degrading leucocyte cAMP (Bevilacqua & Magni, 1993), and in neutrophils the concentration-dependency of the inhibition of PDE IV by nimesulide has an IC_{50} value of 39 μM (Bevilacqua *et al.*, 1994).

These data suggest that nimesulide's total plasma concentration may better reflect its concentration at its sites of action than its free concentration. It is therefore not

inconceivable that the effect of nimesulide on the Ca^{2+} channel current may play some role in reducing myometrial motility *in vivo*. On the other hand, even if this were to be true of indomethacin, its total plasma concentration is much too low for this drug to exert any Ca^{2+} channel blocking action *in vivo*, whereas its COX-antagonistic activity occurs at very low concentrations, so that these probably explain its tocolytic action.

The increased potency of nimesulide at lower pH is also potentially of relevance to the use of nimesulide as a tocolytic agent. Although the pH of the myometrium appears not to have been measured during labour, there is substantial circumstantial evidence for a significant myometrial acidification at this time. It is thought that during labour there is progressive ischemia of the myometrium which is only relieved after delivery. The lactate concentration in the maternal venous circulation increases to 4-5 mM during the final stages of labour (Piquard *et al.*, 1991). This lactate almost certainly originates in the myometrium, since peaks in blood lactate have been observed to occur in association with labour contractions (Marx & Greeve, 1964).

Precisely how far the intracellular pH falls during labour is not known; however small transient intracellular acidifications were associated with spontaneous contractions of very thin strips of rat myometrium even in oxygenated solution (Taggart & Wray, 1994). An *in vivo* study of rats showed that artificially induced ischemia reduced pH from 7.3 to 7.0 (Harrison *et al.*, 1994). Acidification of the myometrium during labour in women should be even more pronounced, since the muscle is thicker and contractions are much more prolonged; more prolonged agonist-induced contractions in rat myometrial strips caused a much greater acidification than spontaneous contractions (Taggart & Wray, 1994). In addition, lactic acid is likely to accumulate in the myometrium, since at delivery there is a reduction in maternal arterial pH which is attributed to a flooding of lactic acid into the circulation (Cohen *et al.*, 1970). Both acidification of the myometrium during the contractions of labour, and the increased level of Ca^{2+} channel inactivation associated with

prolonged slow wave depolarizations, are predicted by the results of this study to greatly enhance the *in vivo* potency of nimesulide as a myometrial relaxant. Nimesulide, which is otherwise a rather weak Ca^{2+} antagonist, may therefore be targeted to myometrial Ca^{2+} channels during labour, in addition to its COX-2 inhibitory action in feto-placental membranes.

Chapter 5

Outward currents in HMSMCs of Pregnant and Non-Pregnant women; characteristics of the fast transient component

5.1. METHODS AND RESULTS

5.1.1. WHOLE CELL CURRENTS

Components of ionic current were initially identified according to their sensitivity to K^+ channel blockers TEA (10 mM) and 4-AP (5 mM). These two pharmacological agents were used in this initial characterisation because they had previously been found to be effective at separating K^+ current components in vascular and visceral smooth muscles (Smirnov and Aaronson, 1992, Smirnov & Aaronson, 1994; Vogalis *et al.*, 1993; Carl, 1995). Most 4-AP sensitive delayed rectifier and A-like K^+ currents are half maximally inhibited by sub-millimolar concentrations of 4-AP, so that 5 mM will provide near complete block (see Table 1.3). Currents were recorded using normal PSS (containing 1.5 mM Ca^{2+}) as the bath solution and high K^+ EGTA-buffered PSS in the pipette. Cells from non-labouring pregnant women at term were compared with those from non-pregnant women of reproductive age (see chapter 2 for details).

The effects of TEA and 4-AP on current voltage-relationships in representative HMSMCs from pregnant and non-pregnant woman, using -80 mV as the holding potential (V_H), are shown in Figs 5.1. and 5.2., respectively. Measurements of current at the peak (10-15 ms) and end of pulse (450-500 ms) at +60 mV in the presence of TEA and 4-AP for both pregnant and non-pregnant HMSMCs are presented in Fig 5.3. Cell hypertrophy causing differences in cell size between non-pregnant and pregnant cells, and hence differences in current amplitude, is known to occur during pregnancy and similar differences in cell capacitance to those described in the present study were found by Wang *et al* (1998) in the rat myometrium. So to correct for these differences (non-pregnant cells were 3-4 fold smaller than pregnant, $C_m = 28.1 \pm 2.8$ pF, $n=10$ in non-pregnant vs. 101 ± 15 pF, $n = 18$ in pregnant), current amplitude (pA) was converted to current density (pA/pF) using the cell capacitance (pF) as an estimate of cell size.

In normal PSS, an outward current was observed at all potentials positive of -40 mV in both pregnant and non-pregnant cells (Panel A, in Fig 5.1. and Fig 5.2.). This current was often very noisy at potentials more positive than 0 mV, was fully activated within 50 ms at potentials above 0 mV and decayed only slightly during 500 ms. The current amplitude was several fold greater in cells from pregnant women compared to non-pregnant (5-10 fold), however when current amplitude was corrected for cell size there was no significant difference between the current densities at +60 mV (Fig 5.3.).

The addition of 10 mM TEA produced significant but not complete inhibition of the outward current in both pregnant and non-pregnant cells (panel B, Figs 5.1. and 5.2.). The amplitude of the TEA sensitive component after 10-15 ms and at the end of pulse (450-500 ms) was estimated by subtracting current in the presence of TEA from current in PSS. Inhibition was on average 32-33 % at the peak and 68-75 % at the end of the pulse at +60 mV. The noisiness of current at +40 mV and +60 mV in PSS was abolished in the presence of TEA. The difference in the size of the TEA-sensitive current at +60 mV after 10-15 ms was significantly smaller than at the end of the pulse in both pregnant cells and non-pregnant cells, suggesting the presence of a slowly activating TEA-sensitive current (Fig 5.3.). The current remaining in the presence of TEA was occasionally composed of transient (rapidly inactivating) and sustained (slowly inactivating) components (as in the example shown in Fig 5.2B).

The further addition of 5 mM 4-AP in the continued presence of 10 mM TEA produced additional block (Panel C, Figs 5.1. and 5.2.). The inhibition was on average greater at the peak than at the end of the pulse, especially in non-pregnant cells, where the transient component of the current revealed in the presence of TEA was always completely inhibited by 4-AP (Fig 3.3.), suggesting the presence of an A-like, rapidly inactivating K^+ current. However, there was also a sustained component to the 4-AP sensitive current especially in

pregnant cells, suggesting the presence of a delayed rectifier-like, slowly inactivating K^+ current.

In the combined presence of 10 mM TEA and 5 mM 4-AP however, there was in many cells a substantial sustained outward current component (Panel C, Figs 5.1. and 5.2.). In pregnant and non-pregnant cells, this 4-AP insensitive current was $21 \pm 2 \%$ and $23 \pm 2 \%$ of the total outward current measured at the end of the pulse for pregnant and non-pregnant cells, respectively (Fig 5.3.). Under these conditions, in all pregnant (7 out of 7) and 2 out of 3 non-pregnant cells examined, a transient inward current component was also revealed at membrane potentials between -40 and +20 mV (Panel C, Figs 5.1. and 5.2., corresponding to I_{Ca} , as described in chapter 3).

Subtraction of the current remaining in the presence of both 5 mM 4-AP and 10 mM TEA from the current present in 10 mM TEA alone provided a picture of the 4-AP sensitive components (Panel D, Figs 5.1. and 5.2.). This also enabled the fast transient 4-AP sensitive component to be separated from the capacitive current artefacts, which, because they were unaffected by the addition of 4-AP, were removed during the subtraction. The amplitude of the sustained component of this 4-AP sensitive “difference current” was significantly larger in pregnant compared to non-pregnant cells (Fig 5.3.).

Under the experimental conditions used here, there are therefore four principle components to the whole cell outward current, namely the sustained TEA-sensitive, transient 4-AP sensitive, sustained 4-AP sensitive and sustained TEA & 4-AP insensitive components, and one inward current component, in pregnant and non-pregnant HMSMCs. However, not all cells possessed all of these components and there was some variability between pregnant and non-pregnant cells. Since there were perhaps not enough cells for some of the apparent differences between pregnant and non-pregnant cells to reach statistical significance (as suggested by data in Fig 5.3.), a qualitative assessment was also

adopted. To this end, the number of cells possessing each of the 4 outward current components are presented below (Table 5.1.).

TABLE 5.1. OCCURRENCE OF OUTWARD CURRENT COMPONENTS IN PREGNANT AND NON-PREGNANT HMSMCS ON THE BASIS OF TEA (10 mM) AND 4-AP (5 mM) SENSITIVITY.

Outward Current Component	Pregnant (total 18 cells)	Non-Pregnant (total 10 cells)
Sustained TEA-sensitive	18 (100 %)	10 (100 %)
Sustained 4AP-sensitive	13 (72 %)	4 (40 %)
Transient 4-AP sensitive	9 (50 %)	8 (80 %)
Sustained 4-AP & TEA insensitive	18 (100 %)	10 (100 %)

FIG 5.1. WHOLE CELL CURRENT IN PREGNANT HMSMCS

Current voltage relationships in a representative HMSMC from a pregnant woman ($C_m = 112.4$ pF). Currents were elicited by 500 ms depolarisations from HP -80 mV to between -80 and +60 mV (20 mV increment at a frequency of 0.1 Hz) in normal PSS (A) and then in 10 mM TEA (B) and both TEA and 5 mM 4-AP (C). The 4-AP sensitive component of the current (derived by subtracting currents in C from currents in B) is shown in D. The 4-AP sensitive component consists of a fast and a slow component. The fast component is made more visible by showing only the first 50 ms of current. Please note the change in vertical scaling between panel A and subsequent panels. The arrows indicate zero current.

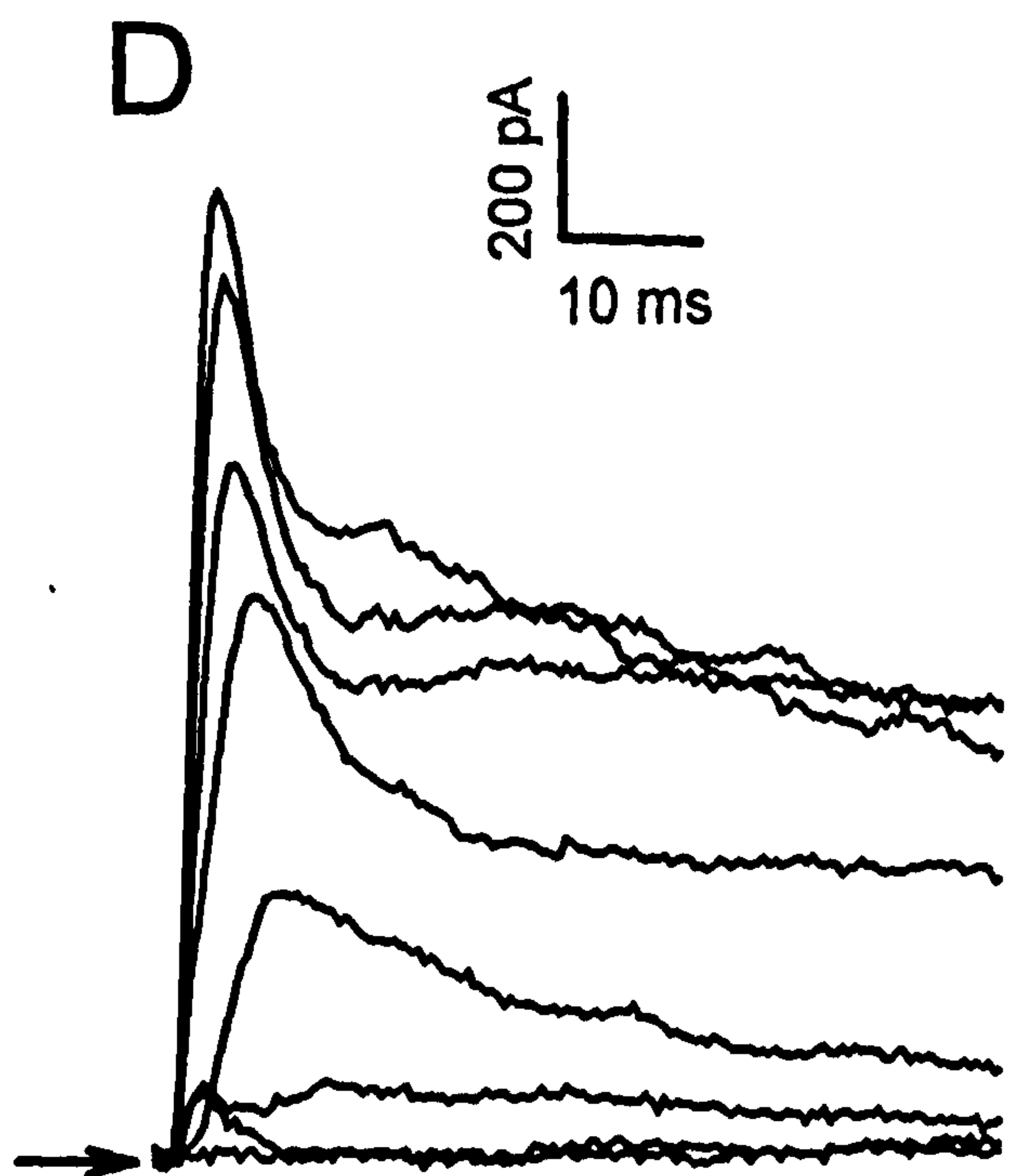
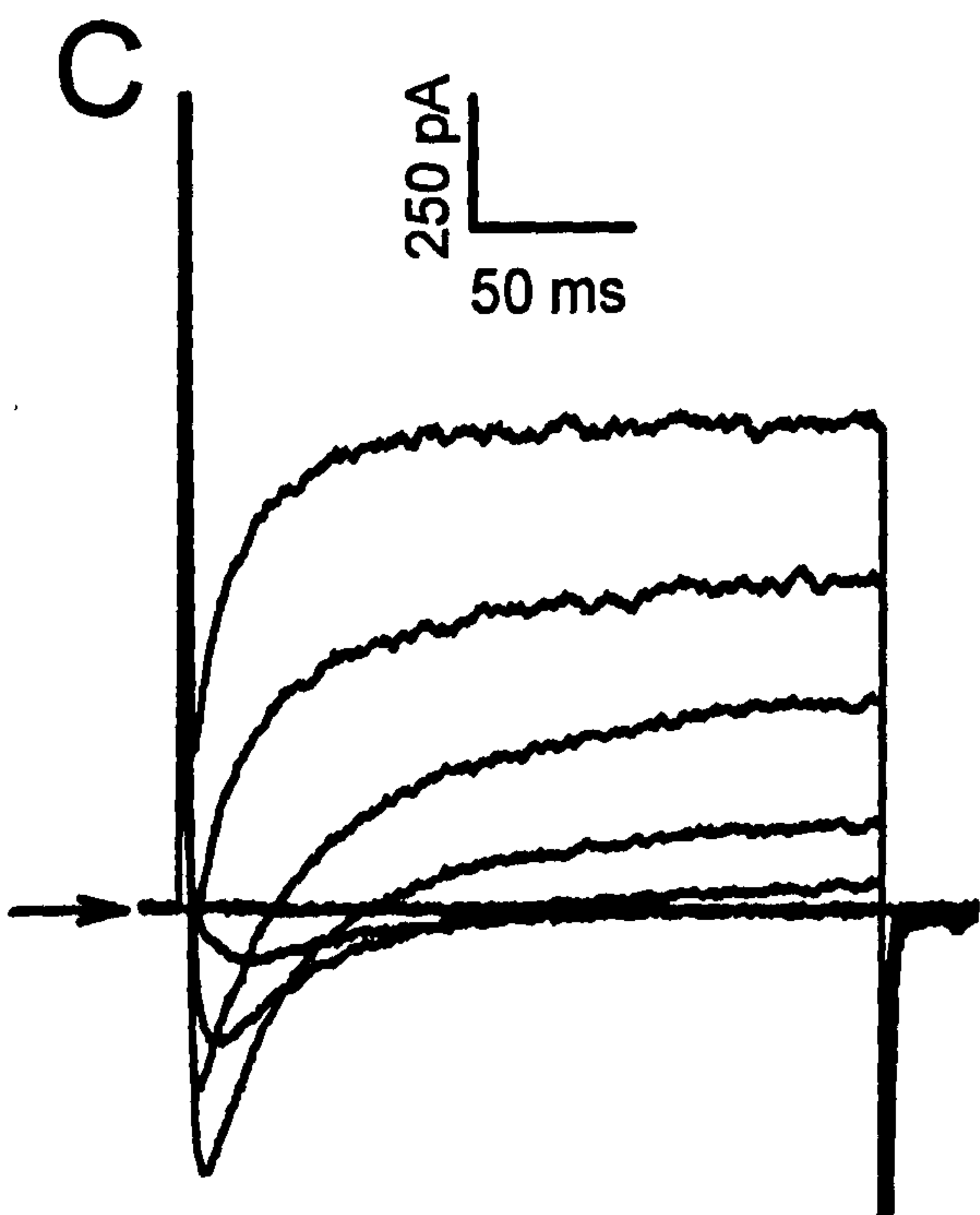
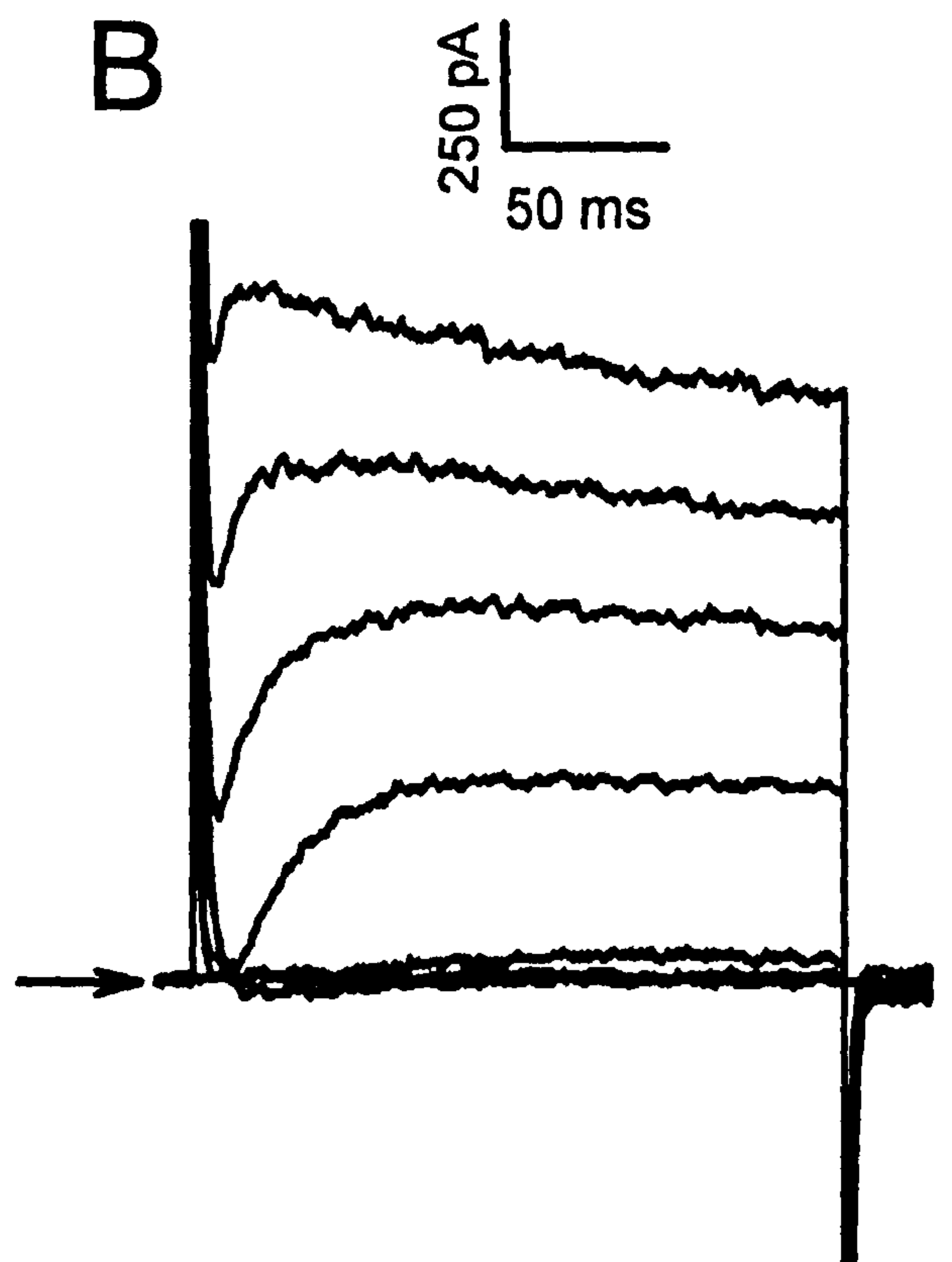
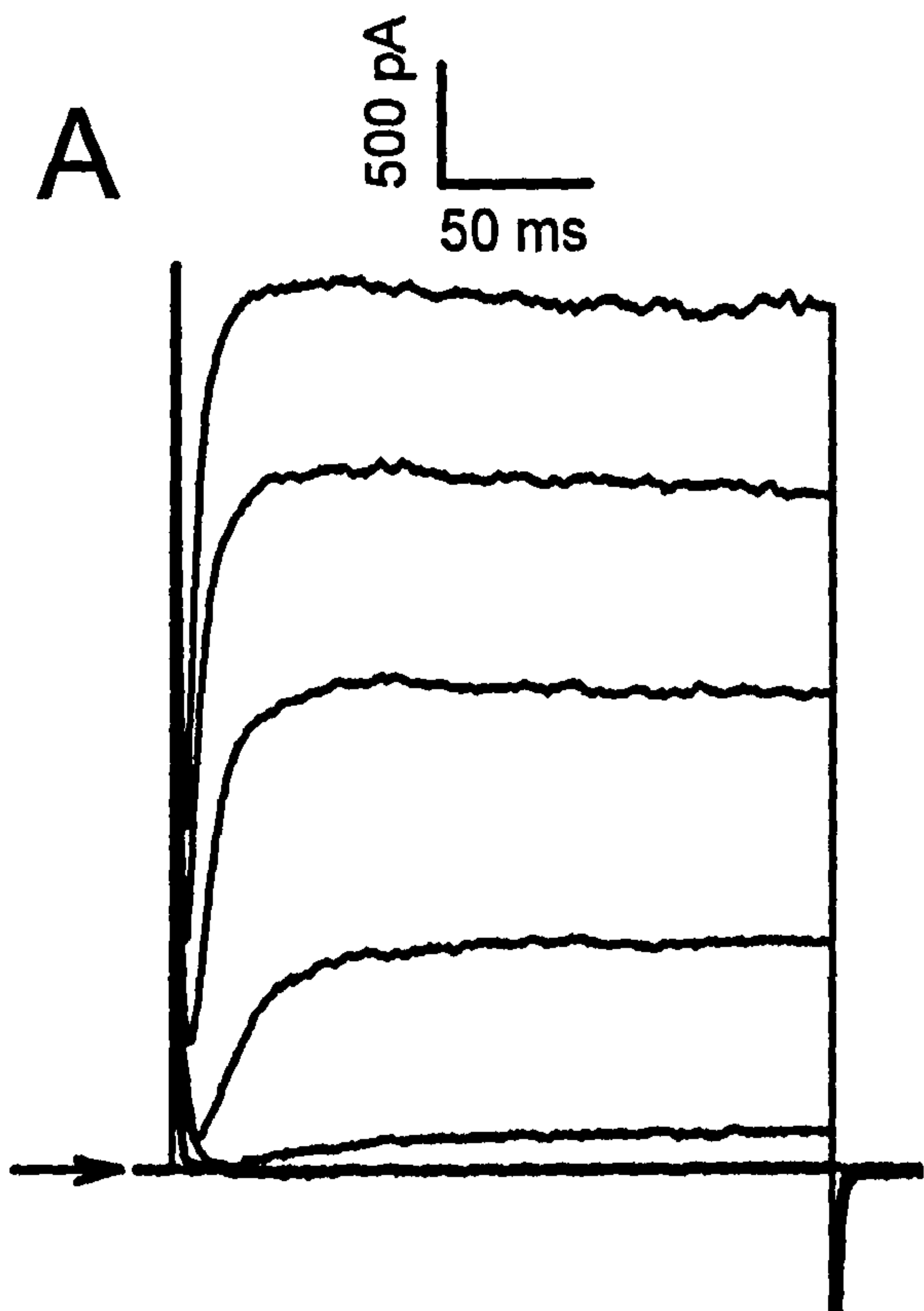


FIG. 5.2. WHOLE CELL CURRENT IN NON-PREGNANT HMSMCS

Current voltage relationships in a representative HMSMC from a non-pregnant woman ($C_m = 27.7$ pF). Currents were elicited by 500 ms depolarisations from HP -80 mV to between -80 and +60 mV (20 mV increment at a frequency of 0.1 Hz) in normal PSS (A) and then in 10 mM TEA (B) and both TEA and 5 mM 4-AP (C). The 4-AP sensitive component of the current (derived by subtracting currents in C from currents in B) is shown in D. The 4-AP sensitive component consists of a fast and a slow component. The fast component is made more visible by showing only the first 50 ms. The arrows indicate zero current.

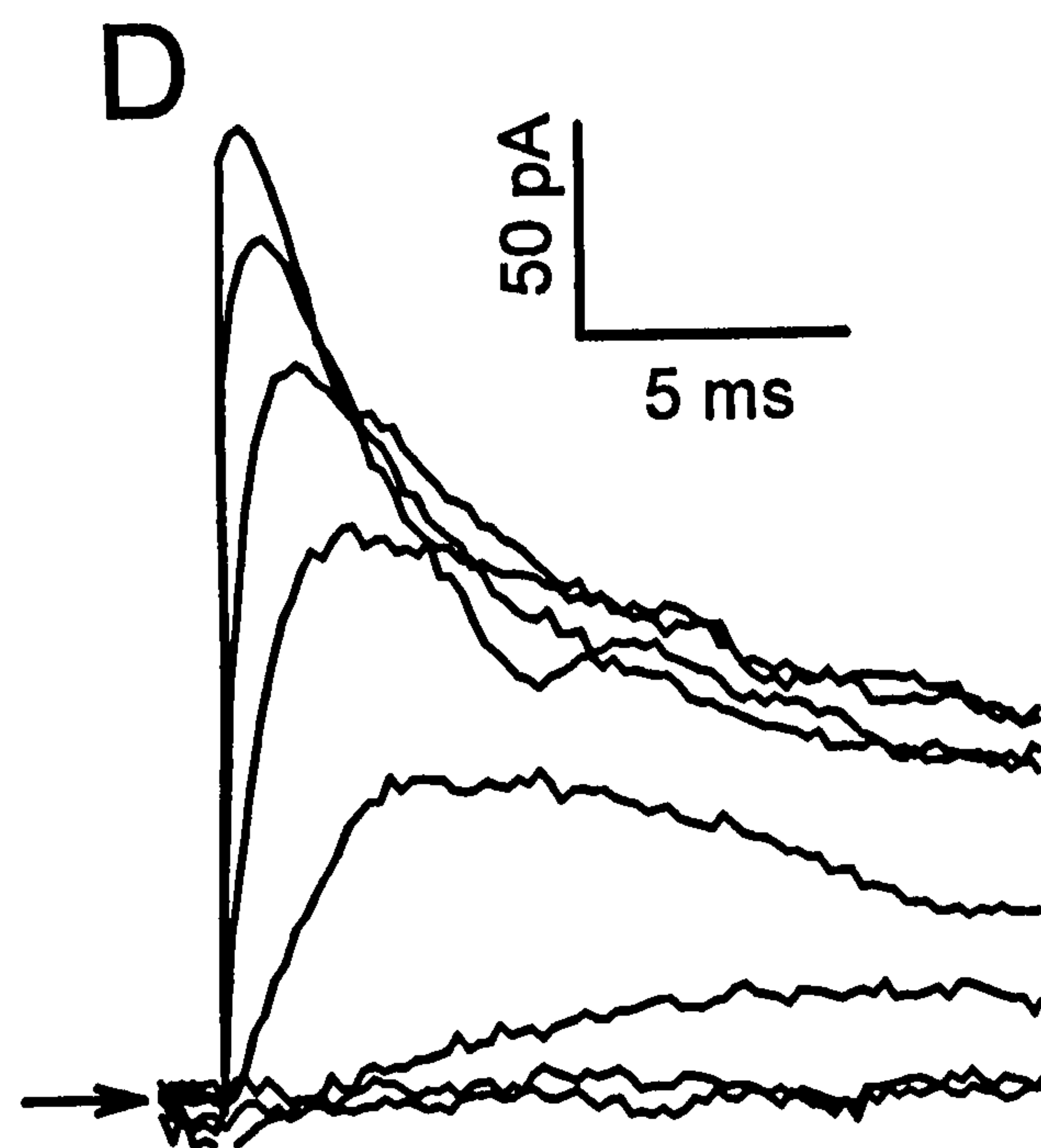
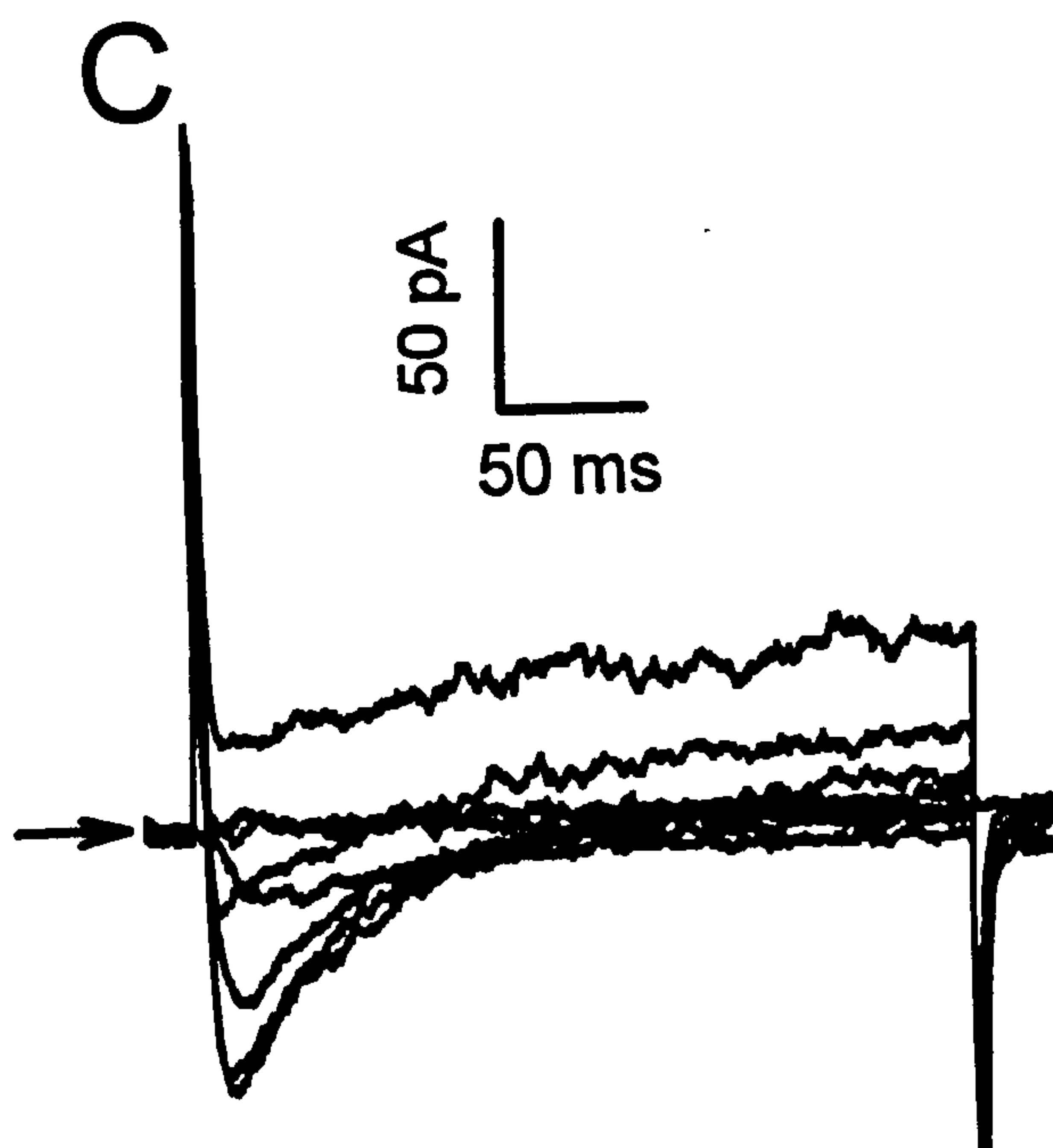
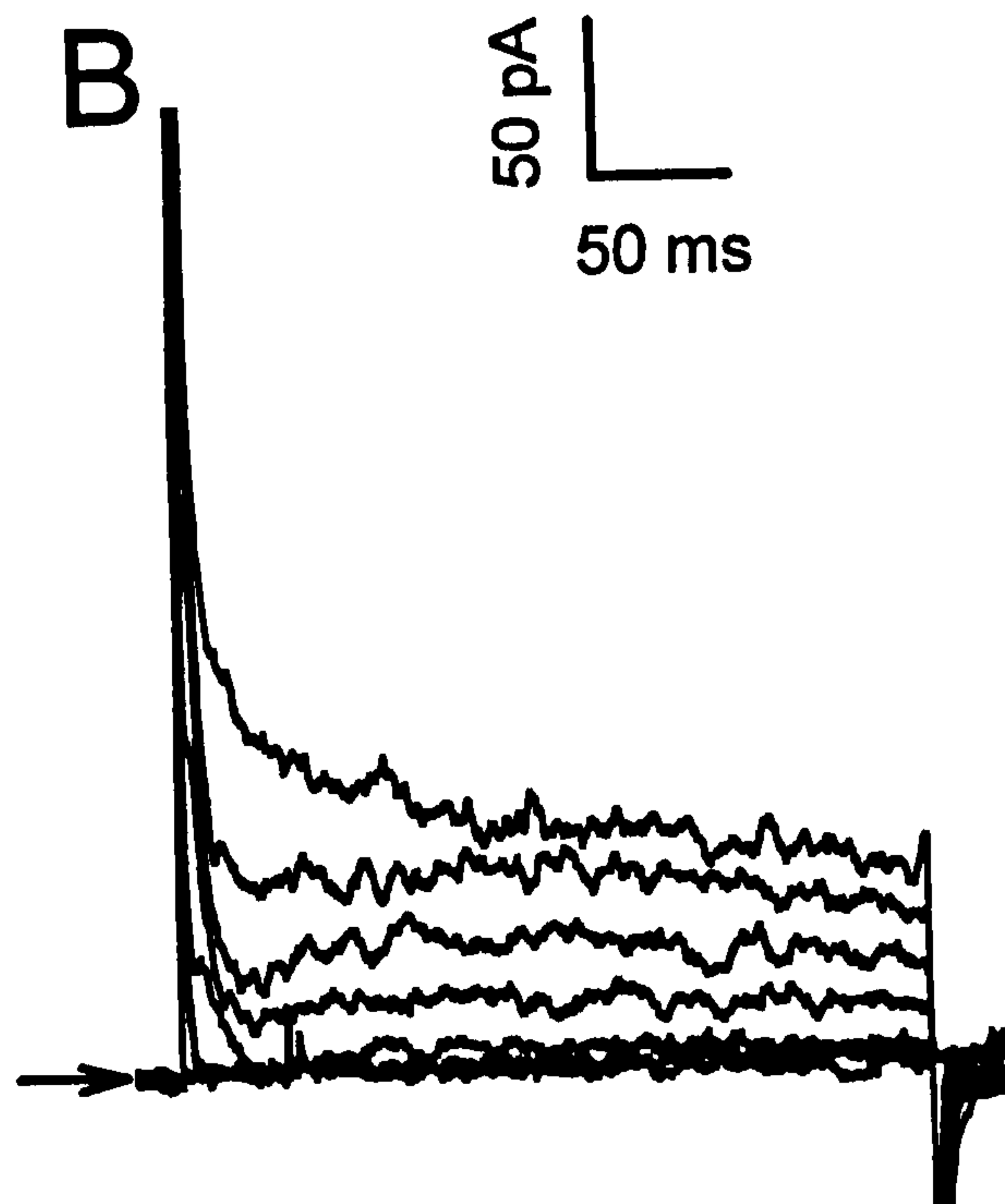
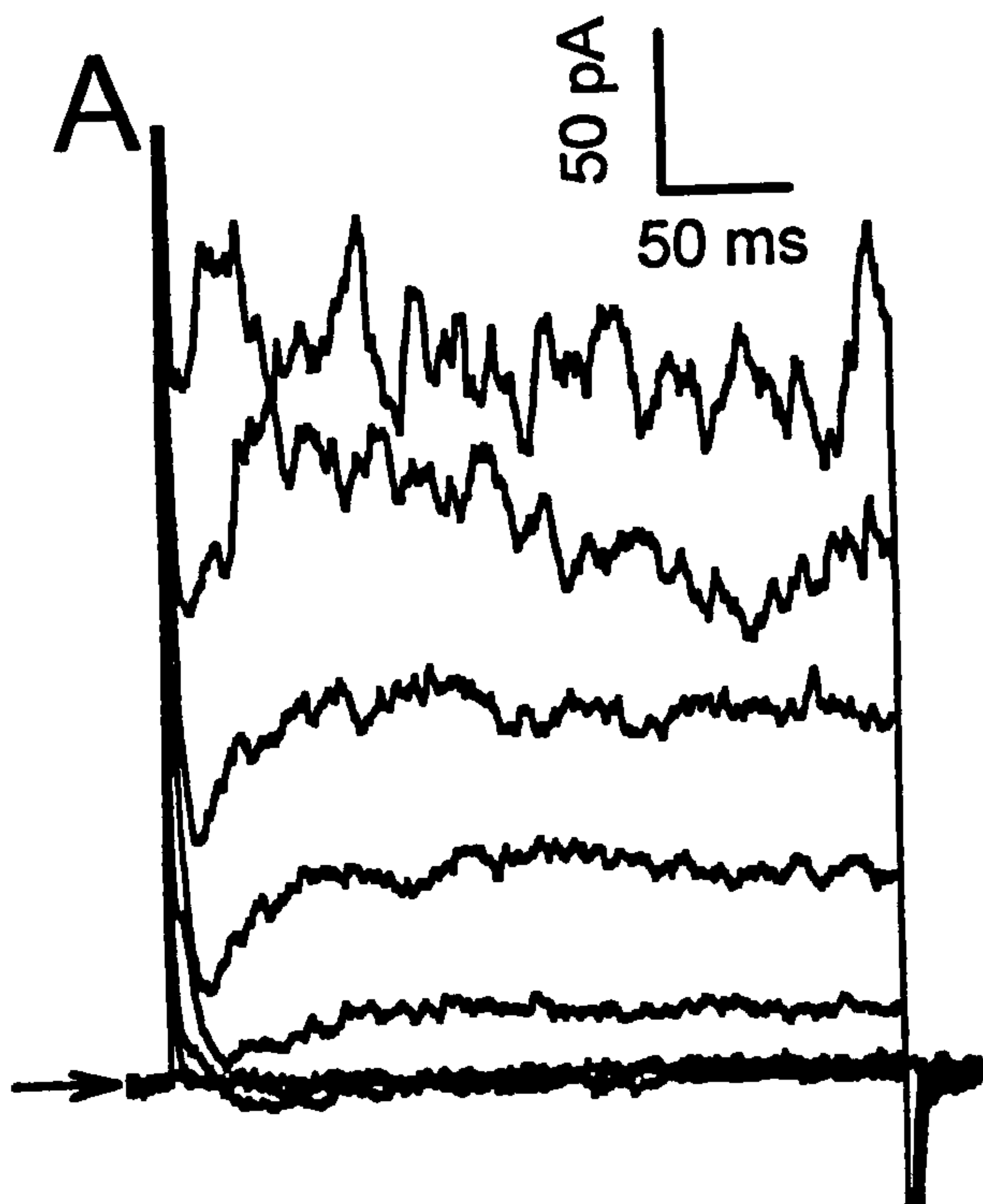
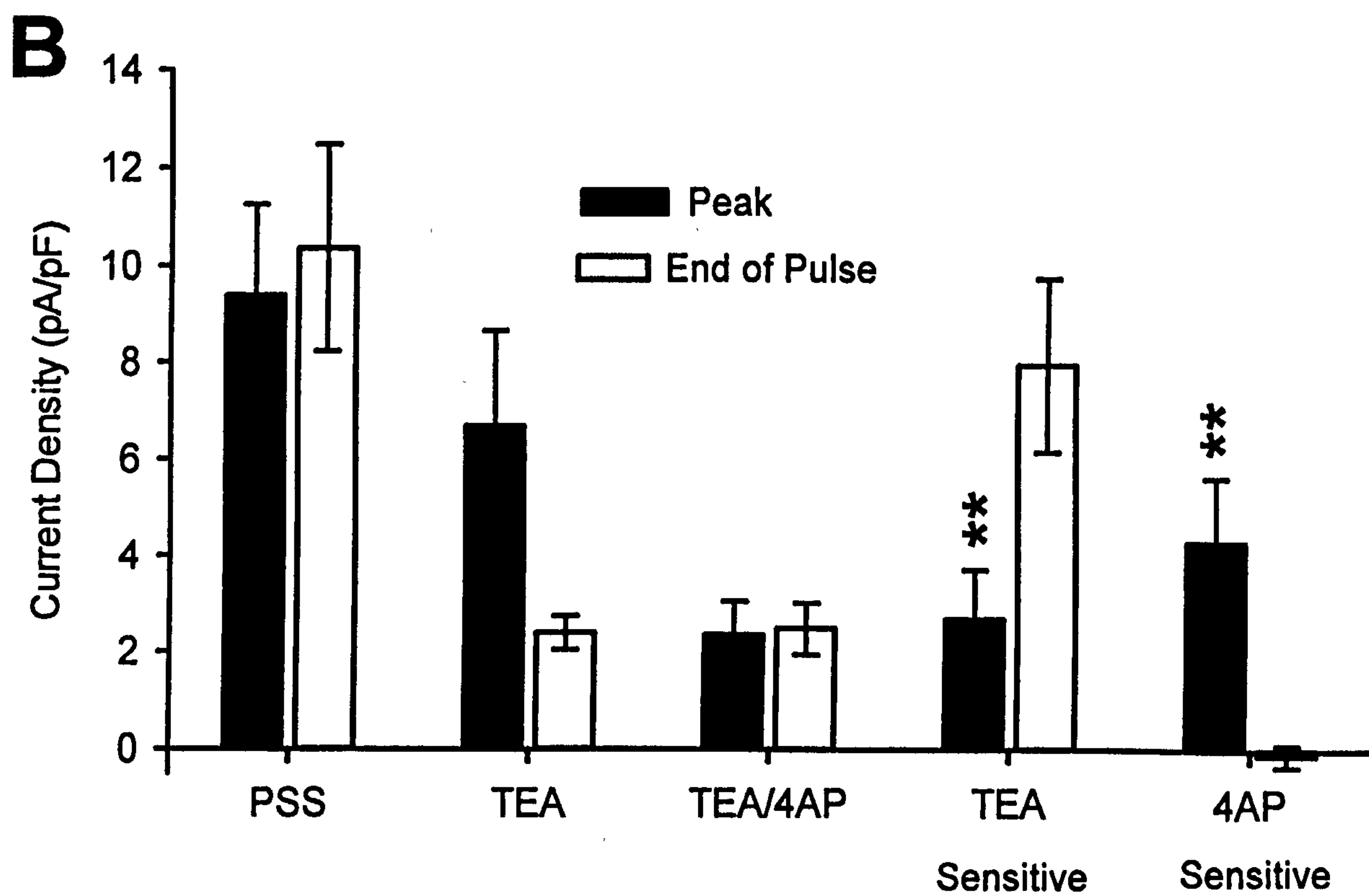
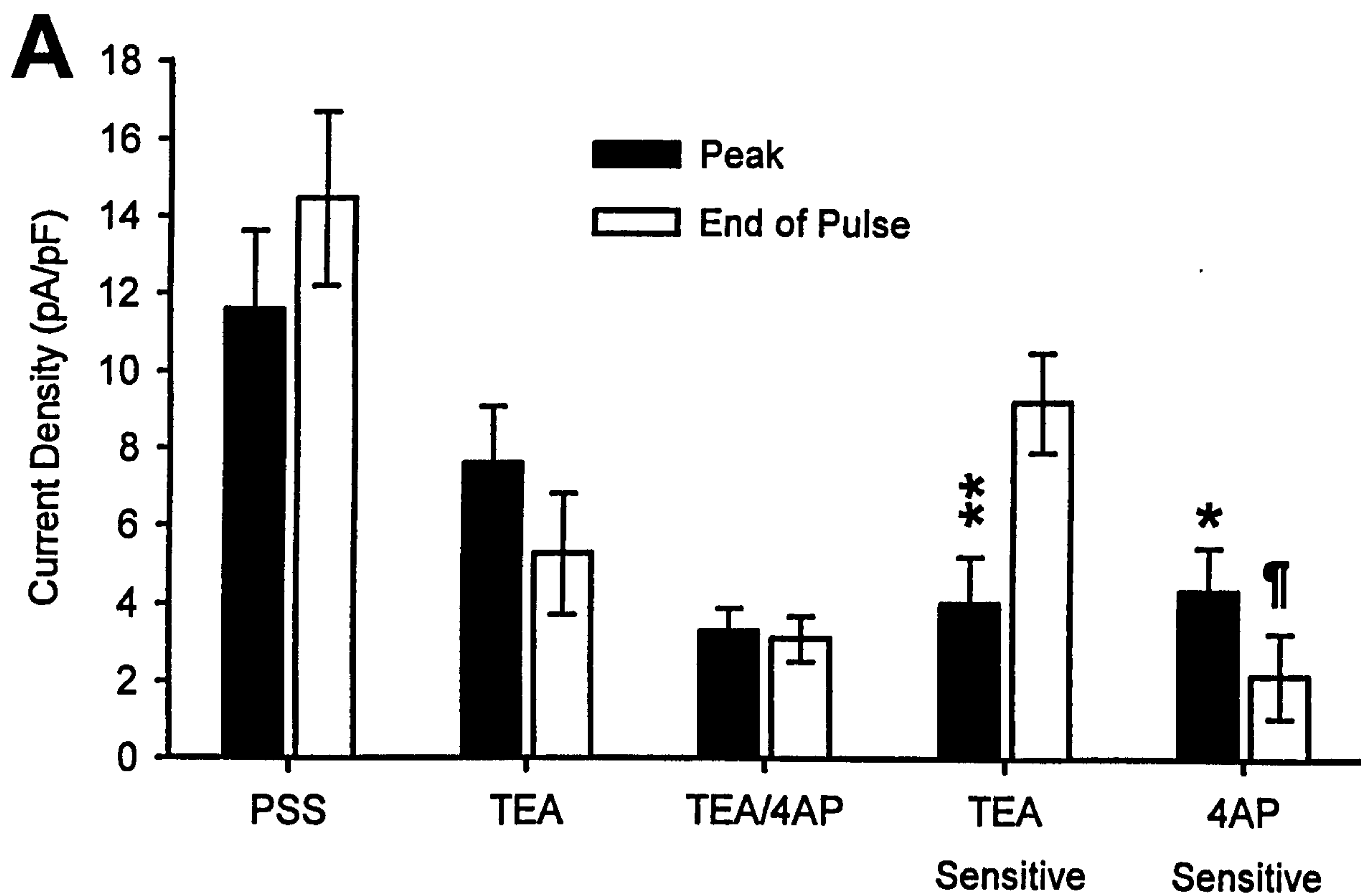


FIG 5.3. COMPARISON BETWEEN PREGNANT AND NON-PREGNANT HMSMCS

Effect of TEA and 4-AP on transient and slow components of whole cell currents in HMSMCs from pregnant (A, n=18 cells) and non-pregnant women (B, n= 10 cells). Transient current amplitude at +60 mV was estimated by measuring at the peak (10-15 ms) and slow current by measuring at the end of the pulse (450-500 ms). Data bars represent the mean \pm S.E.M. of current density. There were significant differences in the size of TEA-sensitive and 4-AP sensitive current components between peak and end of pulse (** $P < 0.0001$, * $P < 0.05$). In addition, the size of the slowly decaying 4-AP sensitive component (ie. the end of pulse measurement) in pregnant cells was significantly greater than in non-pregnant cells ($\nabla P < 0.05$).



5.1.2. CHARACTERISTICS OF THE TRANSIENT 4-AP SENSITIVE CURRENT

As in the two examples given in Figs 5.1. and 5.2., in most cells from both pregnant and non-pregnant HMSMCs an inward current was only observed after the addition of both TEA and 4-AP. This suggests that the transient 4-AP sensitive component of the outward current ($I_{K,A}$) was activating at similar membrane potentials and with similar amplitude and kinetics of activation and inactivation to the inward calcium current (I_{Ca}). To illustrate this further, an example of a cell where both currents are present, and there is minimal contribution of the sustained delayed rectifier current component, is presented in Fig 5.4, showing currents elicited by depolarisations to between -40 mV and +10 mV (at 10 mV increments) in the presence of 10 mM TEA (black traces), the presence of 10 mM TEA and 5 mM 4-AP (blue traces) and the 4-AP sensitive component (red traces). From this example it is clear that I_{Ca} and $I_{K,A}$ overlap quite closely at all potentials shown but that at potentials positive of -30 mV a net fast transient outward current is visible in the presence of TEA, suggesting that $I_{K,A}$ was bigger than and/or activating faster than I_{Ca} . The latter was confirmed by measuring the time taken for the two currents to reach peak amplitude at -20 mV and 0 mV in 4 pregnant HMSMCs. At -20 mV, the time to peak was 13.8 ± 3.6 ms for $I_{K,A}$ and 19.6 ± 2.3 ms for I_{Ca} ($P < 0.05$, paired t-test) and at 0 mV, it was 10.2 ± 2.2 ms for $I_{K,A}$ and 14.7 ± 2.1 ms for I_{Ca} ($P < 0.01$, paired t test).

The voltage-dependence of activation and inactivation of $I_{K,A}$ and its sensitivity to removal of external Ca^{2+} and replacement with Cd^{2+} was also examined in pregnant HMSMCs (Fig 5.5). As described above, $I_{K,A}$ was defined as the transient component of the outward current inhibited by 5 mM 4-AP. Current voltage relationships (steps to between -80 and +20 mV from V_H -80 mV, Fig 5.5A) and availabilities (10 s conditioning pulses to between -100 and +10 mV followed by a test pulse to +60 mV, Fig 5.5B) were therefore performed in the presence of 10 mM TEA, and then TEA and 5 mM 4-AP and the 4-AP

sensitive “difference current” derived. These experiments were performed either with 1.5 mM Ca^{2+} (and 10 mM TEA) or with 0 Ca^{2+} and 0.5 mM Cd^{2+} (and 1 mM TEA, see chapter 6) in the bath solutions. The threshold of activation of $I_{K,A}$ with 1.5 mM Ca^{2+} /10 mM TEA as bath solution was between -80 and -70 mV (7 cells). In the presence of 0.5 mM Cd^{2+} and 1mM TEA however, the threshold of activation in another 2 cells was apparently shifted in the positive direction by at least 30 mV ($n = 2$) (Fig 5.5A). With 1.5 mM Ca^{2+} and 10 mM TEA in the bath solution, following fitting of normalised data to a Boltzmann curve, the inactivation of $I_{K,A}$ was voltage-dependent with $V_{0.5}$ values of -69.5 and -70.0 mV and k of 5.0 and 6.0 mV for the two cells, respectively. $I_{K,A}$ was completely inactivated after conditioning pulses positive of -50 mV. When 0.5 mM Cd^{2+} replaced Ca^{2+} in the bath solution however, $V_{0.5}$ in another 2 cells was -40.1 and -40.6 mV, k was 4.6 and 5.5 mV and the current was completely inactivated after conditioning potentials positive of -20 mV, a rightward shift of approximately 30 mV (Fig 3.5B).

FIG. 5.4. OVERLAP OF $I_{K,A}$ WITH I_{Ca} IN HMSMCS

In a non-pregnant cell where the slow 4-AP sensitive current and the 4-AP insensitive currents are minimal, overlap of inward current (I_{Ca} , blue traces) and transient 4-AP sensitive outward current ($I_{K,A}$, red traces) is apparent at all membrane potentials between -40 and +10 mV (from V_H -80 mV), as shown. The net current in TEA (black traces) shows a net fast transient current at potentials positive of -30 mV. A similar pattern was observed in 4 pregnant cells (see text). The dotted lines represent zero current.

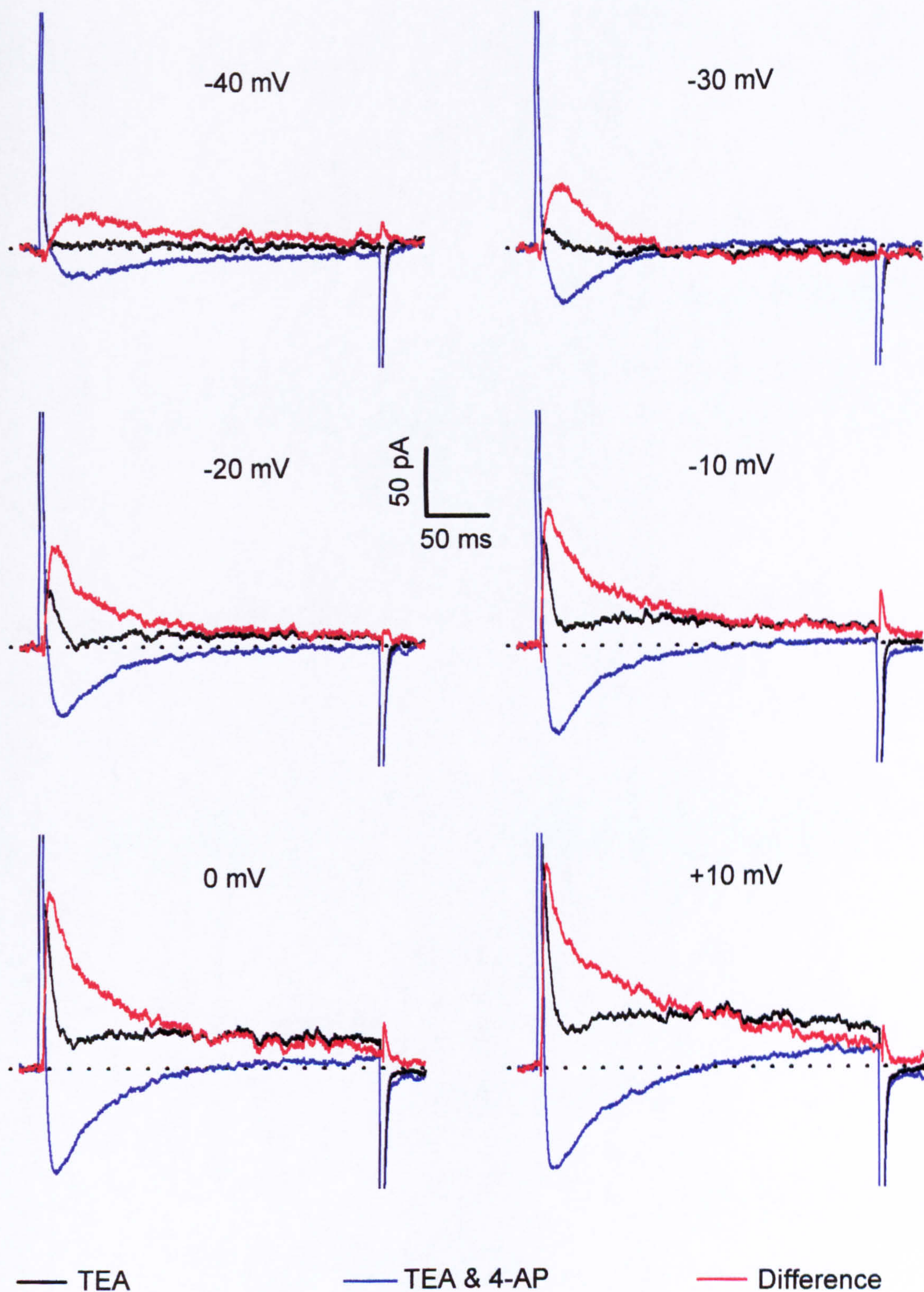
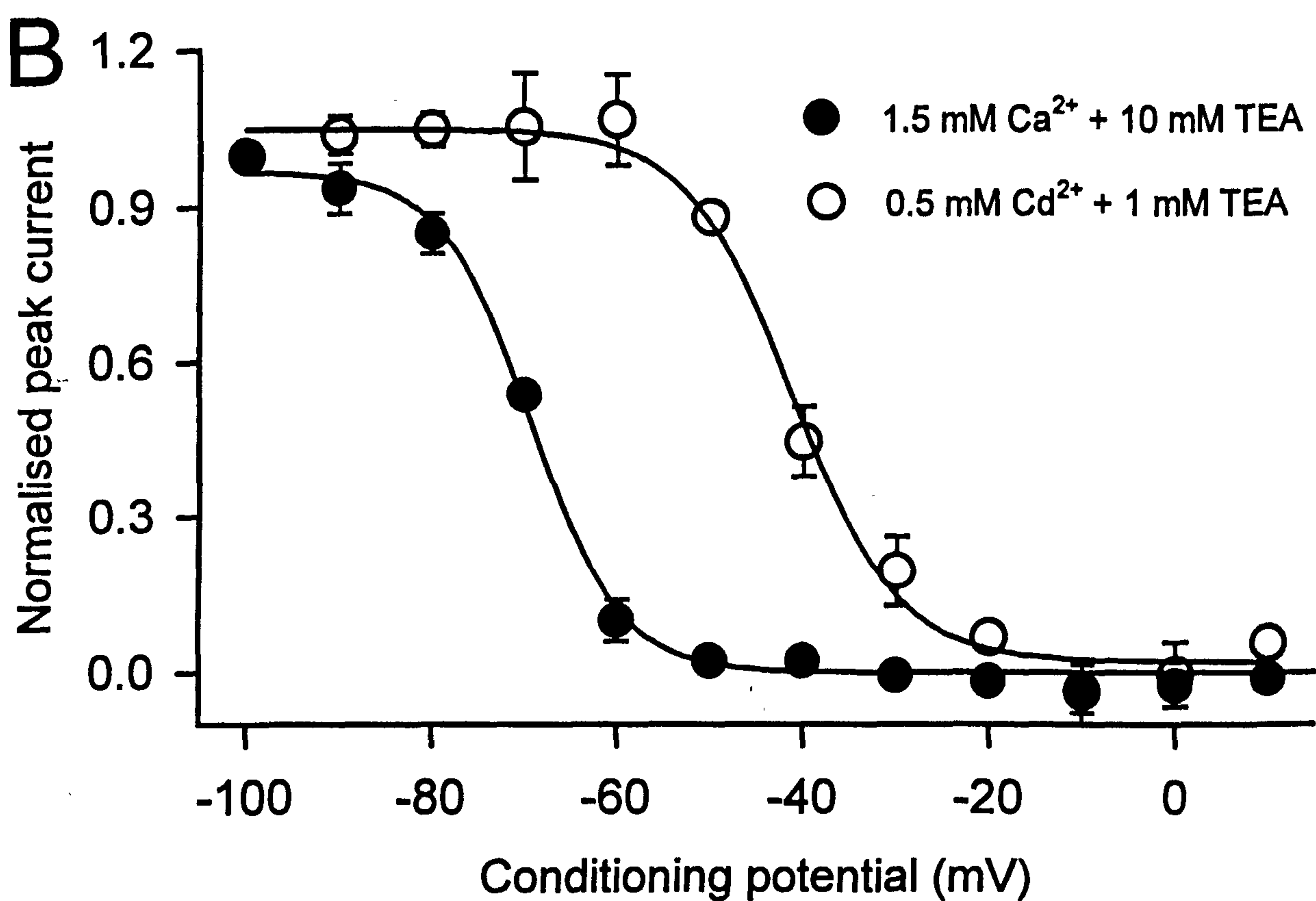
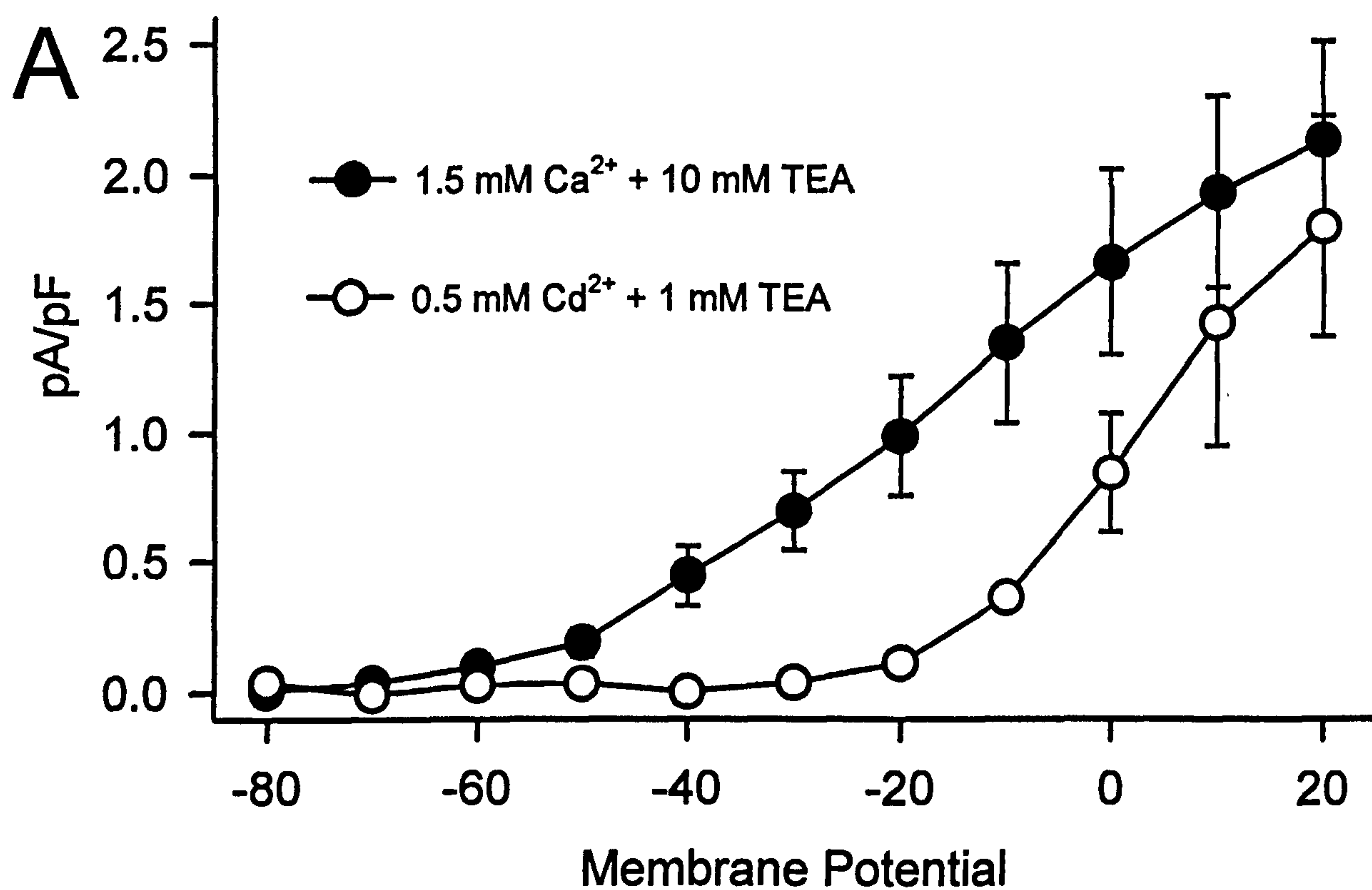


FIG. 5.5. CURRENT-VOLTAGE RELATIONSHIPS AND AVAILABILITIES OF $I_{K,A}$

Current voltage relationships (A) and availabilities (B) of $I_{K,A}$, defined as the transient component of the 4-AP sensitive current, in pregnant HMSMCs. A: Mean and SEM current densities at 20-30 ms from V_H -80 mV, with either 1.5 mM Ca^{2+} (●, n=7) or 0.5 mM Cd^{2+} in the bath (○, n=2). B: Mean and SEM of currents measured at +60 mV following 10 s conditioning pulses to between -100 and +10 mV normalised to the current following the -100 mV conditioning pulse, with either 1.5 mM Ca^{2+} (●, n=2) or 0.5 mM Cd^{2+} (○, n=2) in the bath.

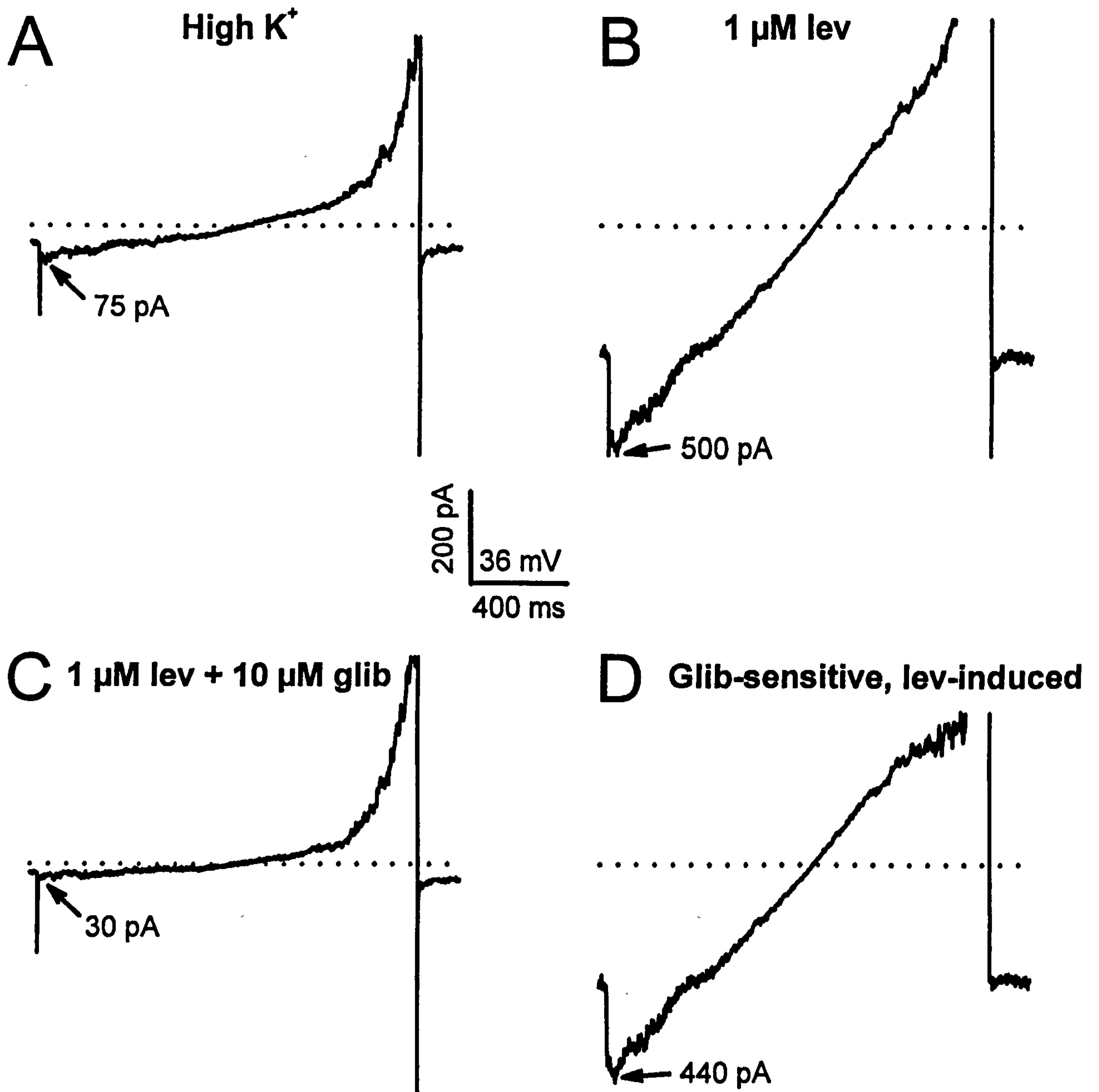


5.1.3. GLIBENCLAMIDE-SENSITIVE K⁺ CURRENT IN HMSMCS

The existence of a glibenclamide-sensitive, ATP dependent K⁺ current ($I_{K,ATP}$) in pregnant HMSMCs was investigated. To isolate this current, cells were dialysed with normal high K⁺ pipette solution, but without any ATP (to maximise baseline $I_{K,ATP}$), and perfused with Ca²⁺-free PSS, containing high K⁺ (140 mM KCl replacing NaCl) as the bath solution. This was in order to shift the K⁺ reversal potential close to 0 mV, thus enabling $I_{K,ATP}$ to be more easily measured as an inward current at -100 mV, where other K⁺ currents are negligible. 1 mM TEA was also present in the bath solution to block $I_{K(Ca)}$.

The example given in Fig 5.6 shows a 1 second current ramp to between -100 mV and +50 mV from a holding potential of -60 mV. As expected for a K⁺ current under these conditions, the current was inward at -100 mV (Fig 5.6A, indicated by arrow), outward at +50 mV, with reversal near 0 mV. The inward current at -100 mV was increased 6-7 fold by the addition of 1 μ M levcromakalim, an activator of $I_{K,ATP}$ (Quayle *et al.*, 1997) (Fig 5.6B, indicated by arrow) and, in the continued presence of levcromakalim, was blocked by 10 μ M glibenclamide, a blocker of $I_{K,ATP}$ (Quayle *et al.*, 1997) (Fig 5.6C, indicated by arrow). The glibenclamide-sensitive current, derived by subtracting current in the presence of both levcromakalim and glibenclamide from current in the presence of levcromakalim alone, is shown in D. The effects of both levcromakalim and glibenclamide were fully reversible on washout (not shown). In order to determine whether $I_{K,ATP}$ could still be measured under a normal K⁺ gradient and with ATP in the pipette, the same voltage protocol was applied to another cell from the same myometrial sample, using 5 mM ATP in the pipette and normal low K⁺ PSS containing 10 mM TEA in the bath. In that cell, 10 μ M glibenclamide had no effect on current amplitude at -100 mV or at +50 mV.

Fig 5.6. Glibenclamide-sensitive current in HMSMCs



1 second duration current ramps to between -100 and +50 mV from HP -60 mV in a pregnant HMSMC. **A:** high K^+ PSS containing 1mM TEA. **B:** the addition of 1 μM levcromakalim (lev). **C:** the addition of 10 μM glibenclamide (glib). **D:** Glibenclamide-sensitive current, derived by subtracting current in **C** from current in **B**. Current amplitude at -100 mV is indicated by the arrows. The dotted lines represent zero current.

5.2. DISCUSSION

5.2.1. TEA SENSITIVE CURRENT

The ionic currents of HMSMCs are composed of TEA and 4-AP sensitive components and components insensitive to the combination of both drugs. The noisy TEA-sensitive component almost certainly represents the large conductance (hence the noisiness) calcium-activated potassium current ($I_{K(Ca)}$) which has been extensively characterised in rat and human myometrium (Toro *et al.*, 1990b; Perez *et al.*, 1993; Anwer *et al.*, 1993; Khan *et al.*, 1993; Perez & Toro, 1994; Meera *et al.*, 1995; Khan *et al.*, 1997; Wang *et al.*, 1998). This component was of similar magnitude in both pregnant and non-pregnant cells. This contrasts with the recent study in rat myometrium (Wang *et al.*, 1998), where $I_{K(Ca)}$ formed a considerably larger component of the whole current in cells from non-pregnant rats, when cell size was corrected for, and where its activation was shifted in the hyperpolarised direction in non-pregnant cells ($V_{0.5} = +39$ mV) compared to cells from pregnant rats ($V_{0.5} = +63$ mV).

5.2.2. TRANSIENT 4-AP SENSITIVE CURRENT ($I_{K,A}$)

The transient “A-like” component of the 4-AP sensitive current ($I_{K,A}$) has also been described previously in human (Erulkar *et al.*, 1993) and in rat myometrium (Miyoshi *et al.*, 1991; Erulkar *et al.*, 1994; Wang *et al.*, 1998). The voltage dependencies of activation and inactivation of $I_{K,A}$, with 1.5 mM Ca^{2+} in the bath solution in the present study (activation threshold and inactivation $V_{0.5}$ both around -70 mV), differ from those attributed to the A-like current in cultured leiomyoma cells from non-pregnant women, with 5 mM Ca^{2+} in the bath solution (activation threshold \sim -40 mV and inactivation $V_{0.5}$ of -24 mV, Erulkar *et al.*, 1993) or in 17 β -estradiol treated cells from immature rat uterus with 2.5 mM Ca^{2+} in the bath solution (threshold \sim -50 mV and $V_{0.5}$ of -48 mV, Erulkar *et al.*, 1994), but were similar to those of the I_{TO} described in freshly dispersed non-pregnant

rat myometrial cells, with 1 mM Ca^{2+} in the bath solution (threshold and $V_{0.5}$ both < -70 mV, Wang *et al.*, 1998). These differences may be due to the different $[\text{Ca}^{2+}]_o$ used in the four studies, since Wang *et al.*, (1998) showed that in the non-pregnant rat myometrium, the activation and inactivation of I_{TO} were both shifted in the positive direction by raising $[\text{Ca}^{2+}]_o$ from 1 to 30 mM. Indeed, A-currents in other tissues have been shown to be sensitive to the concentration of most extracellular divalent cations, such as Cd^{2+} , which, when replacing a similar concentration of Ca^{2+} in the bath solution, causes large depolarising shifts (between 10 and 30 mV) in the voltage-dependence of both activation and inactivation in guinea-pig ureter smooth muscle (Imaizumi *et al.*, 1990), opossum esophageal circular muscle (Akbarali *et al.*, 1995) and rat and human ventricular myocytes (Stengl *et al.*, 1998).

In pregnant and non-pregnant cells where both $I_{\text{K,A}}$ and I_{Ca} were present, the two currents overlapped at potentials between -40 mV and +10 mV. In most of these cells there was no net inward current in the absence of 4-AP but rather a net rapid transient outward current that increased in amplitude as the membrane potential was increased. This current existed mostly because $I_{\text{K,A}}$ activated faster than I_{Ca} , as indicated by the faster time to peak of $I_{\text{K,A}}$ at -20 and 0 mV than of I_{Ca} . A similar overlap of these two currents and faster time to peak of $I_{\text{K,A}}$ has also been found in guinea-pig colonic smooth muscle cells (Vogalis *et al.*, 1993). Furthermore, in the guinea-pig colon (Vogalis *et al.*, 1993) as well as guinea-pig ureter (Imaizumi *et al.*, 1990) and opossum esophageal circular muscle (Akbarali *et al.*, 1995), the addition of 4-AP under current clamp conditions caused a leftward shift in the upstroke of the action potential as well as causing an overall depolarisation. It is believed therefore that in these tissues the A-current may be available at the resting membrane potential and may activate before and simultaneously with I_{Ca} , thus delaying or even preventing action potential spike generation. 4-AP has also been shown to depolarise strips of pregnant rat myometrium (Wilde & Marshall, 1988), but its effect on the upstroke of the action potential was not examined. The described electrophysiological properties of

$I_{K,A}$ are similar to those in the other smooth muscles discussed above and a proportion of the channels responsible for this current would be expected to be available at and perhaps contribute to the resting membrane potential (around -50 mV in pregnant human myometrium, see table 1.1.).

In the present study, $I_{K,A}$ occurred more frequently in cells from non-pregnant women (80 %) than in cells from pregnant women (50 %), but, probably because of the number of cells examined, when this difference was examined in terms of mean current density, it was not statistically significant. A more pronounced difference was found by Wang et al. (1998) in rat myometrium where I_{T0} was found in 50 % of non-pregnant myocytes but was very rare in pregnant cells. The lack of significance in the present study may also have been due to contamination by the 4-AP sensitive delayed rectifier current which was more abundant in pregnant cells, although this is unlikely since $I_{K,A}$ activated so much faster than the delayed rectifier.

There is some evidence to suggest however that expression of $I_{K,A}$ may be influenced by steroid hormones. For example, estrogen treatment in immature rats reduced the incidence of myometrial cells expressing $I_{K,A}$ from 79 % to 30 % (Erulkar *et al.*, 1994). These effects are potentially of physiological relevance because activity of the uterus is associated with circulating steroid hormone levels (Csapo, 1956; Marshall, 1962; Abe, 1970). In pregnant sheep for example, estradiol rises sharply at or around delivery then falls sharply back below gestational levels post-partum (Thorburn & Challis, 1979; Verhoeff *et al.*, 1985b) In humans a similar pattern occurs, although the rise in estrogen level is more gradual (Berg & Kuss, 1992). Alternatively, Erulkar *et al.*, 1993 suggest that $I_{K,A}$ may have a role in cell growth and proliferation, since in human non-pregnant myometrium it was much more abundant in leiomyoma cells (a benign myometrial tumor) than in normal differentiated cells.

5.2.3. “DELAYED RECTIFIER” CURRENTS

Most cloned delayed rectifier K⁺ channel currents (Table 1.3) and many native delayed rectifier type K⁺ currents are sensitive to 4-AP, with EC₅₀ values in the micromolar range. In the present study, the resistance of a substantial component of the outward current to block by the combination of 10 mM TEA and 5 mM 4-AP therefore suggests the presence of either a single voltage-gated current with low sensitivity to 4-AP (EC₅₀ ~5 mM), or two currents, one with relatively high sensitivity (EC₅₀ < 1 mM) and one with relatively low sensitivity to 4-AP (EC₅₀ >> 5 mM). The presence of a TEA and 4-AP-insensitive component of the delayed rectifier current in human and rat myometrium has been implied by previous studies, but it has not been characterised in any great detail (Miyoshi *et al.*, 1991; Erulkar *et al.*, 1993; Inoue *et al.*, 1993; Erulkar *et al.*, 1994).

In the present study, the 4-AP sensitive component of the delayed rectifier current was significantly greater in pregnant cells than in non-pregnant cells, assuming that this difference represents a shift in the relative expression of channel types, rather than a change in 4-AP sensitivity of a single channel type. The physiological relevance of this is unknown, as is whether delayed rectifier currents are influenced by steroid hormones in a manner similar to that of $I_{K,A}$. The only other voltage-gated K⁺ current known to be influenced by steroid hormones is the slow delayed rectifier minK/K_vLQT1 (Boyle *et al.*, 1987b), which is relatively insensitive to 4-AP.

In a preliminary study published in abstract form, using the same experimental conditions as described in this chapter, inactivation of the sustained 4-AP-insensitive component of the delayed rectifier current was shown to be voltage-dependent, with a relatively negative $V_{0.5}$ (-67 mV) (Smirnov *et al.*, 1995). The pharmacological and electrophysiological characterisation of the delayed rectifier current, following the assumption that there are two distinct voltage-gated currents, one 4-AP sensitive and the other 4-AP insensitive, is

described and discussed in more detail in chapter 6, in which care was taken to remove or diminish any influence of I_{Ca} , $I_{K(Ca)}$ and $I_{K,A}$ on current characteristics.

5.2.4. GLIBENCLAMIDE-SENSITIVE CURRENT

When current ramps from -100 mV up to +50 mV were applied to cells with symmetrical K^+ in the pipette and bath solutions, a shallowly sloping current was present, being inward at -100 mV and reversing close to 0 mV. In a cell where ATP was excluded from the pipette solution, this current was linearly enhanced by levcromakalim and inhibited by glibenclamide, suggesting the presence of an ATP-sensitive K^+ current ($I_{K,ATP}$) in HMSMCs. In another cell with 5 mM ATP in the pipette, this current was not present. Direct measurement of K_{ATP} current has not previously been reported in the myometrium, but its presence has been implied by the action of drugs selective for this channel on myometrial contraction *in vitro*. In those studies, spontaneous or OT-induced contractions of pregnant human and rat myometrium were inhibited by levcromakalim, aprikalim, pinacidil (all activators of K_{ATP} channels), and these effects were partially or completely antagonised by the blocker glibenclamide (Cheuk *et al.*, 1993; Morrison *et al.*, 1993; Piper & Hollingsworth, 1995; Khan *et al.*, 1998).

The relaxant effect of channel openers proves that the channels are present but does not prove that they are normally involved in controlling the resting membrane potential or action potential repolarisation. However, glibenclamide was shown to increase spontaneous contractions in non-pregnant but not pregnant human myometrium *in vitro* (Cheuk *et al.*, 1993), suggesting that expression of myometrial K_{ATP} channels may change in response to pregnancy, and in a more recent *in vitro* study in strips of pregnant human myometrium, glibenclamide did not significantly affect the amplitude or decay of the after-hyperpolarisation induced by PGE_2 or $PGF_{2\alpha}$ (Parkington *et al.*, 1999). Even if the K_{ATP} channel is down-regulated in pregnancy, however, its ATP sensitivity may be important in

labour when the myometrium becomes hypoxic and ATP levels will be expected to be lowered. Under these conditions $I_{K,ATP}$ may be activated sufficiently to induce hyperpolarisation, thus providing periods of relaxation between contractions, as has been demonstrated in coronary and mesenteric vascular smooth muscle (Quayle *et al.*, 1997).

Chapter 6

Voltage-Gated Potassium Channel Currents in HMSMCs of Pregnant Women

6.1. METHODS AND RESULTS

6.1.1. ISOLATION OF “DELAYED RECTIFIER” K^+ CURRENTS

All experiments described in this chapter were performed with freshly dispersed HMSMCs from pregnant women who were not in active labour. As described in chapter 5, there is an outward delayed rectifier current in HMSMCs that appears to have two components, a component sensitive to 5 mM 4-AP and a component insensitive to 5 mM 4-AP. The detailed characterisation of these two currents required that all other currents (I_{Ca} , $I_{K(Ca)}$ and $I_{K(A)}$) be minimised or completely removed if possible. Ca^{2+} -free PSS was used throughout to abolish I_{Ca} and 0.5 mM $CdCl_2$ was added to the bath solution to completely block Ca^{2+} channels thus eliminating the possibility that K^+ efflux through those channels were contributing to the outward current at positive membrane potentials. These two changes also served to diminish the amplitude of $I_{K(Ca)}$. The majority of the remaining $I_{K(Ca)}$ was eliminated by the addition of 1 μ M paxilline, a selective blocker of BK_{Ca} channels (Sanchez & McManus, 1996), or 1 mM TEA, to the bath solution. The TEA concentration was lowered from 10 to 1 mM to take into account the possibility that the delayed rectifier currents were TEA sensitive, and the sensitivity of the voltage-gated currents, as well as $I_{K(Ca)}$, to TEA is presented later in this chapter (Fig 6.5.).

As is shown in chapter 5, $I_{K(A)}$ is also sensitive to Cd^{2+} . At 0.5 mM, because of the large rightward shift in the activation of $I_{K(A)}$, Cd^{2+} caused considerable block of the current at membrane potentials negative of 0 mV but less so at more positive potentials (Fig 5.5.). In the majority of subsequent experiments under these conditions, no further A-like currents were observed.

Using the above described modified bath solutions, the addition of 5 mM 4-AP was found to have different actions to those described in chapter 5. In some cells, 4-AP caused

considerable inhibition of the current, leaving very little outward current, while in others there was very little inhibition of the substantial outward current or even a slight increase when 4-AP was applied. This differential action of 4-AP suggested the idea that there were at least two distinct components to the delayed rectifier current which could be distinguished by their 4-AP sensitivity, and which varied in their relative proportions between cells. Subsequent experiments supported this possibility.

6.1.2. AVAILABILITY OF THE VOLTAGE-GATED K⁺ CURRENT

One of the important characteristics of the delayed rectifier is its tendency to inactivate at depolarised membrane potentials. Therefore, using Ca²⁺ free PSS containing 0.5 mM CdCl₂ and 1 mM TEA in the bath solution, availability of the delayed rectifier K⁺ current was investigated with the following experimental protocol. The membrane potential was held at -80 mV and 10 second conditioning pre-pulses to between -100 mV and +10 mV were applied, followed by a 200 ms test pulse to +60 mV (at 0.03 Hz). This protocol was then repeated in the presence of 5 mM 4-AP.

Fig. 6.1A shows the currents elicited by the +60 mV test pulse following conditioning voltages to between -100 mV and +10 mV, in one cell. The current began to inactivate after conditioning potentials positive of -90 mV, and showed little further inactivation positive of -40 mV. The addition of 5 mM 4-AP did not on average affect the overall current amplitude following the -100 mV conditioning potential, but appeared to increase the component of the current that inactivated following the conditioning pulse to -40 mV, without significantly affecting the half-inactivation value (Fig. 6.1B). By contrast, in another morphologically similar cell, the same voltage-protocol revealed a current that only began to inactivate when conditioning potentials positive of -50 mV were applied, and which was almost completely inactivated at 0 mV (Fig. 6.1C). This current was almost completely blocked by 5 mM 4-AP (not shown, but see Fig. 6.3).

A similar correlation between the voltage range of current availability and 4-AP sensitivity appeared to exist in most cells. This correlation was further investigated using several approaches. Firstly, cells were divided into 2 groups on the basis of a strong (>70 % block) or weak (little or no inhibition, or an increase, in current amplitude) block of the current by 5 mM 4-AP. The availability of the current was assessed in each cell by measuring current amplitude at the end of a 200 ms test pulse to +60 mV, which followed a conditioning potential between -100 and +10 mV. Fig. 6.1D illustrates the mean availability of the current in the 4-AP sensitive (filled squares) and insensitive (filled circles) groups of cells. The open circles represent the availability of the 4-AP-insensitive cells in the presence of 5 mM 4-AP. All data points have been normalised to the current amplitude observed after the conditioning step to -100 mV. In the absence and presence of 4-AP, the amplitude of the test pulse after the -100 conditioning step was 1095 ± 337 and 1269 ± 429 pA, respectively (non-significant, $n = 6$).

The data illustrated in Fig. 6.1D were fitted to the Boltzmann function in order to derive the parameters $V_{0.5}$ (half-inactivation potential), k (slope factor) and A (fraction of the current not inactivated). In the absence of 4-AP, $V_{0.5}$ of the current in the 4-AP insensitive cells was -65 ± 3 mV, the non-inactivating component (A) was 36 ± 2 % and k was 8 ± 2 (n = 6). In the presence of 5 mM 4-AP, $V_{0.5}$ in these cells was -68 ± 3 mV, the non-inactivating component was 37 ± 4 % and k was 6 ± 2 mV (n = 6). Conversely, the $V_{0.5}$ of the current in the group of cells sensitive to 4-AP was -30 ± 1 mV. The non-inactivating component of the current in this group of cells was 15 ± 4 % and k was 5 ± 1 mV (n = 6). The differences in the half inactivation potentials and size of the non-inactivating component between the two groups of cells were highly significant ($P < 0.001$).

The low threshold current which was not inhibited or slightly increased by 5-mM 4-AP, and which reached its maximal extent of inactivation negative of -30 mV (filled circles in Fig. 6.1D), shall hereafter be referred to as I_{K1} . The high threshold current, which was

inhibited by 4-AP and inactivated between -50 mV and 0 mV (filled squares in Fig. 6.1D) will be termed I_{K2} .

The small degree of overlap which existed between the potential ranges over which I_{K1} and I_{K2} inactivated suggested that it would be possible to investigate the properties of each current by utilising different holding potentials. The following 3-step inactivation protocol based on the distinctive inactivation properties of these currents was therefore applied. Current was recorded at the end of a 200 ms test pulse to +60 mV firstly after holding at -80 mV, then after a 10 second pre-pulse to -40 mV in order to inactivate I_{K1} , and then after a 10 second pre-pulse to 0 mV to inactivate I_{K2} . The amplitude of I_{K1} (in cells where I_{K1} was the predominant current) was therefore estimated as the current remaining after the -40 mV pre-pulse subtracted from the current present after the -80 mV holding potential, and the amplitude of I_{K2} (in cells where I_{K2} was the predominant current) was estimated from the current remaining after the pre-pulse to 0 mV subtracted from the current remaining after the pre-pulse to -40 mV.

In the 14 cells where the 3 step inactivation protocol was carried out in the absence and presence of 5 mM 4-AP, the cells were clearly divided into two groups (Fig. 6.2A). In one group (I_{K1}) current was inactivated by pre-pulses to -40 mV and was either unaffected or enhanced by 5 mM 4-AP ($n = 7$, filled squares). No further inactivation was observed in these cells when the conditioning potential was set at 0 mV (open squares). In the other group (I_{K2}) current was almost completely inhibited by 5 mM 4-AP, and was only slightly inactivated at -40 mV (filled circles, $n = 7$). The current in this group of cells was then almost completely inactivated by prepulses to 0 mV (open circles).

The 3-step inactivation protocol described above was carried out in 42 cells. Fig. 6.2B depicts the extent to which the conditioning potential of -40 mV inactivated the outward current, relative to the inactivation recorded at 0 mV in each cell. These results show that

two distinct groups of cells could be distinguished on the basis of the degree to which the conditioning potential-sensitive component was inactivated at -40 mV. The peak on the left represents cells in which the outward current showed relatively little inactivation at -40 mV (*ie* cells where I_{K2} was dominant), while the peak on the right represents cells in which the outward current was markedly inactivated at -40 mV (cells where I_{K1} was dominant). Note that the 4 bars on the extreme right represent cells in which the current was somewhat larger after the conditioning step to 0 mV than it was after the conditioning step to -40 mV. This effect may have been due to some activation of the minor residual component of $I_{K(Ca)}$ or some other unidentified current during the conditioning step to 0 mV (see discussion).

The membrane capacitance of the cells comprising each peak in Fig. 6.2B was calculated from the area under the capacitative artefacts as an indication of the cell surface area, and therefore cell size (Smirnov & Aaronson, 1994). The cell capacitance was 99.9 ± 9.6 pF in the 24 cells in which I_{K1} predominated, and 110.6 ± 12.6 pF in the 18 cells in which I_{K2} was dominant; these values were not significantly different. As far as could be determined, all biopsies were taken from the same region of the myometrium from women at term who were not in active labour, and under light microscopy there were no obvious morphological differences between the two groups of cells. There was no preferential expression of either I_{K1} or I_{K2} in cells from any one biopsy, each isolation often producing some cells with only I_{K1} and some with only I_{K2} . Only on very rare occasions were cells found that expressed an equal amplitude of both I_{K1} and I_{K2} .

FIGURE 6.1. INACTIVATION OF DELAYED RECTIFIER K⁺ CURRENTS

A-C: Examples of the 4-AP insensitive current recorded at +60 mV following 10 second conditioning pre-pulses to between -100 and 0 mV (at 10 mV increments) in the absence (**A**) and presence (**B**) of 5 mM 4-AP. **C** shows the inactivation of the 4-AP sensitive current for potentials between -90 and +10 mV in a different cell under identical conditions as in **A**. Currents following the pre-pulses to -80, -40 and 0 mV are indicated by arrows. The dotted lines represent zero current.

D: Mean and S.E.M. end of pulse measurements of the currents represented in **A-C**, normalised to the current elicited following the -100 mV conditioning pre-pulse. Points show the 4-AP insensitive current in the absence (●, n = 6) and presence of 5 mM 4-AP (○, n = 6) and the 4-AP-sensitive current in the absence of 4-AP (■, n = 5).

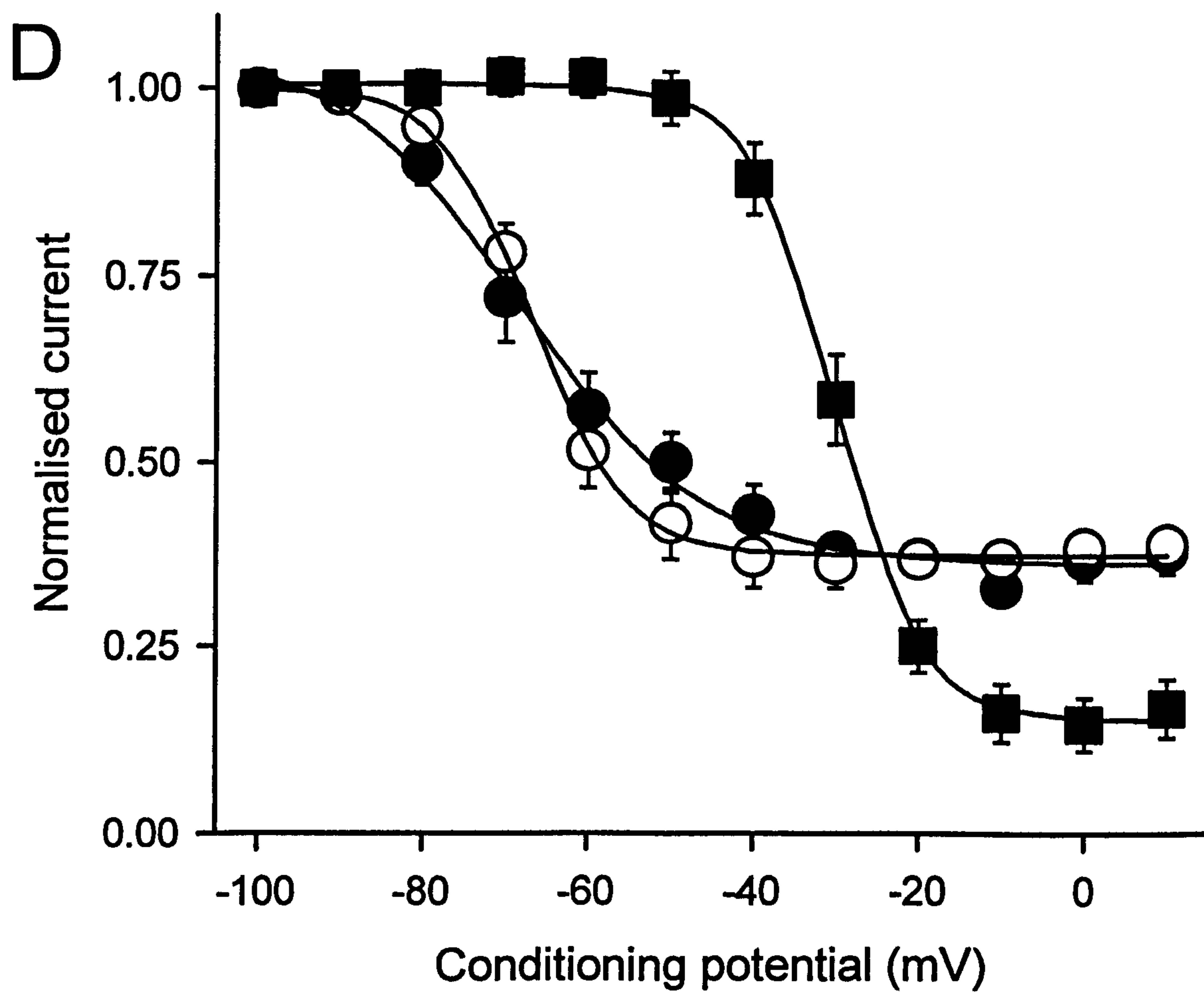
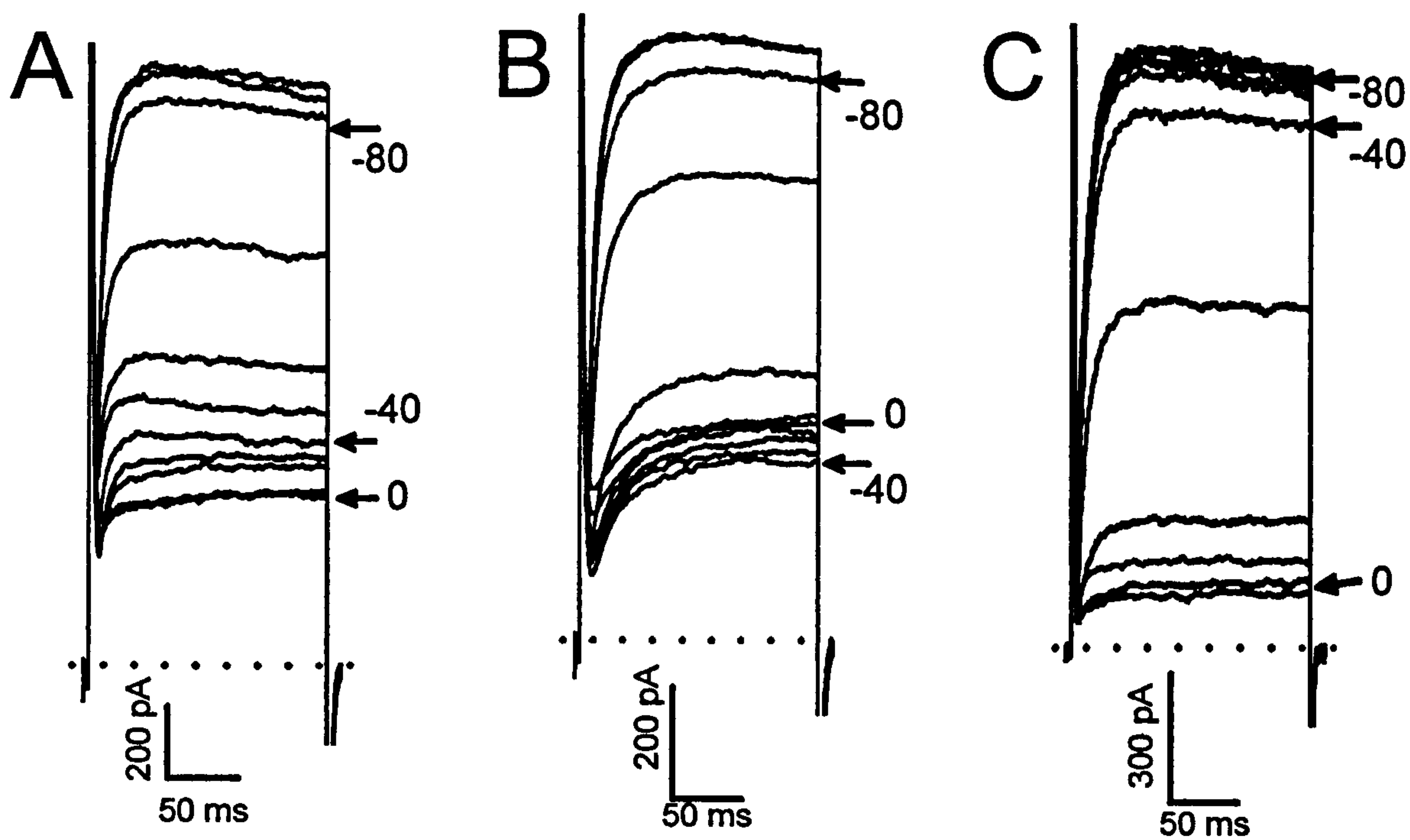
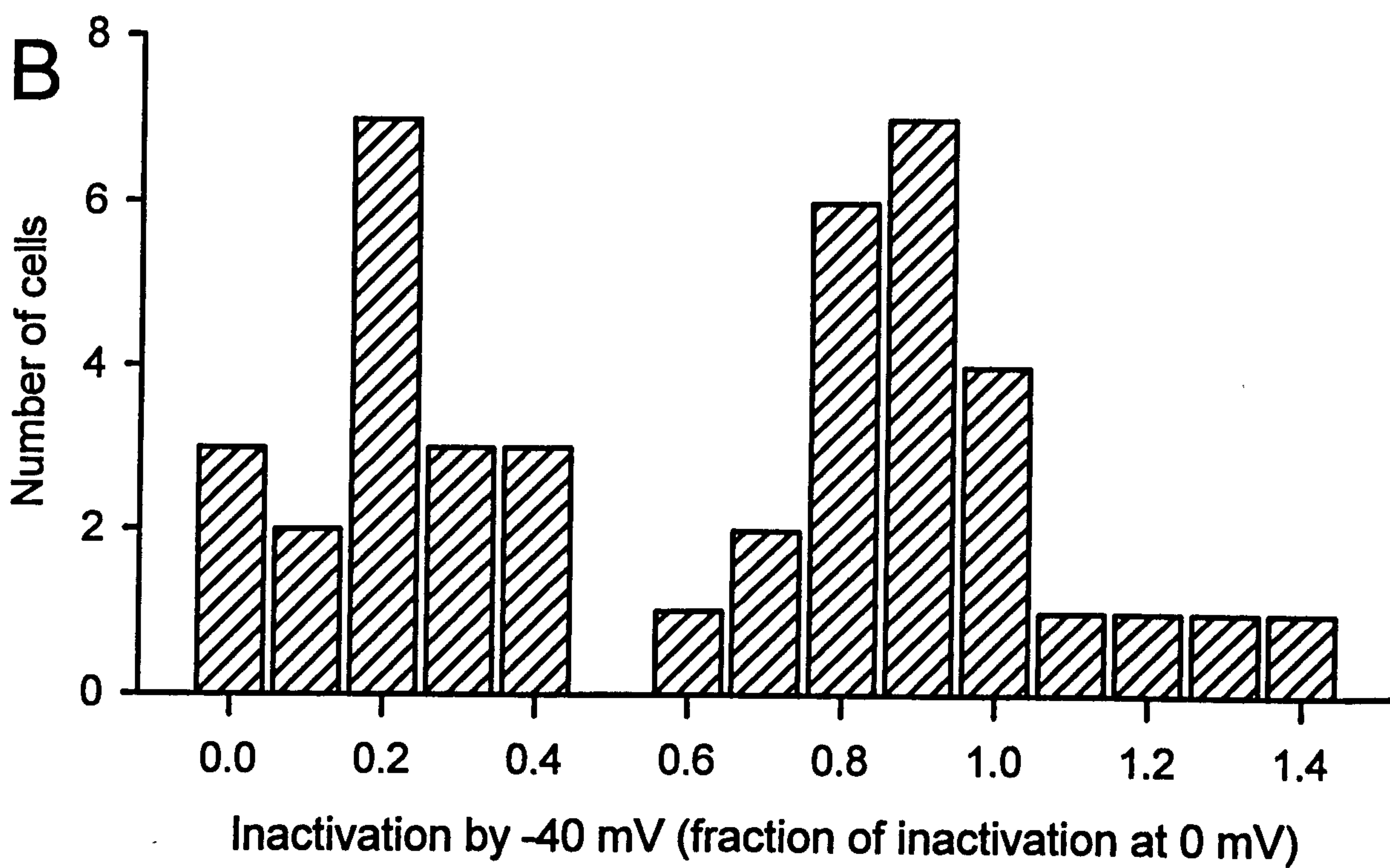
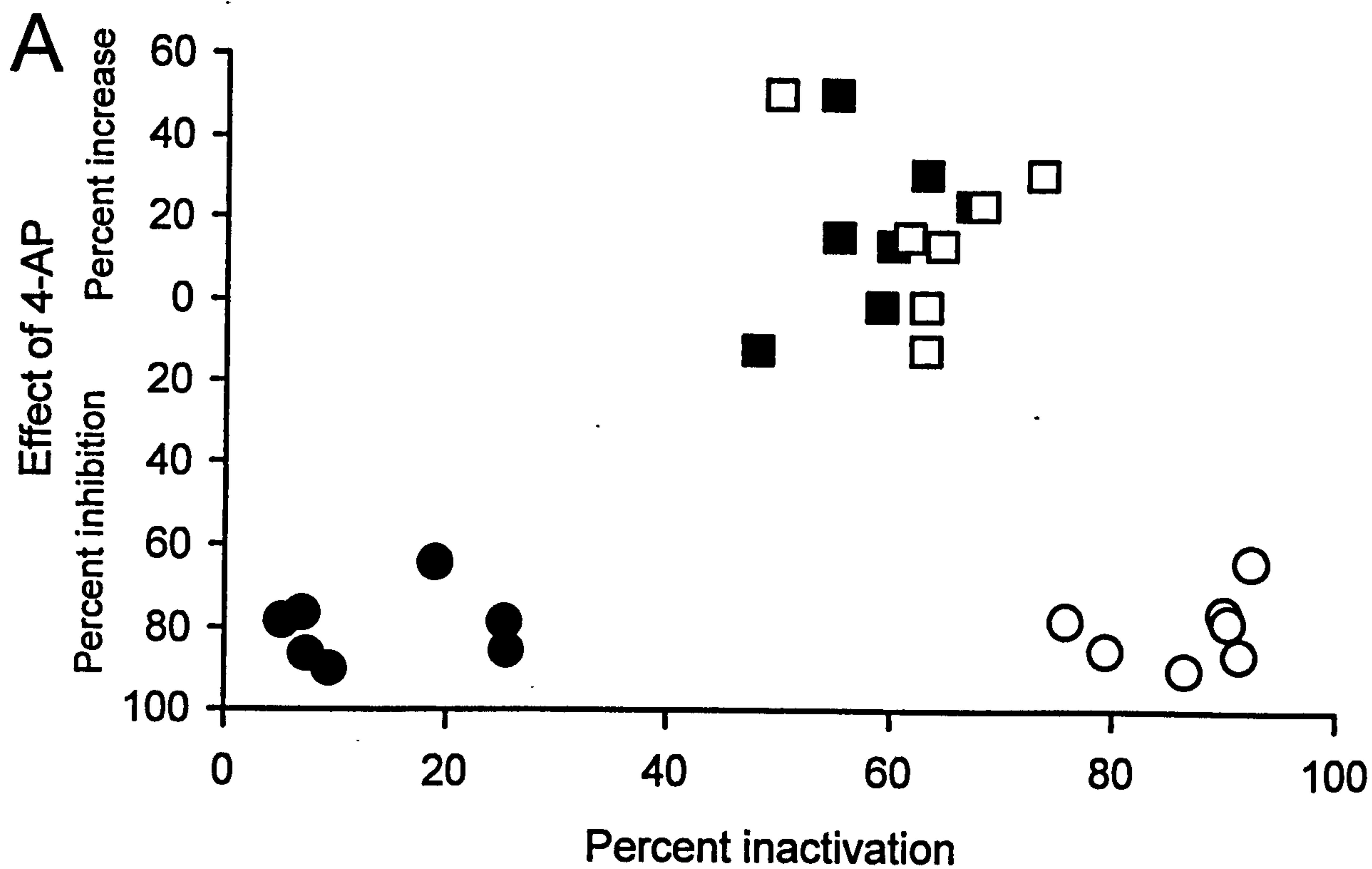


FIGURE 6.2. DIFFERENTIAL DISTRIBUTION OF I_{K1} AND I_{K2}

A: Relationship between inactivation and sensitivity to 5 mM 4-AP for I_{K1} ($n = 7$) and I_{K2} ($n = 7$) currents. The percentage of current at +60 mV inactivated after a 10 second conditioning pre-pulse to either -40 mV (I_{K1} , ■; I_{K2} , ●) or 0 mV (I_{K1} , □; I_{K2} , ○) is plotted against the percentage change in peak current amplitude caused by the application of 5 mM 4-AP during a step to 0 mV from a holding potential of -80 mV. **B:** Frequency distribution of inactivation of the current at +60 mV by a 10 s conditioning pulse to -40 mV in 42 cells, expressed as a fraction of the total inactivating component using the degree of inactivation by a pre-pulse to 0 mV as maximum. Data are binned at intervals of 0.1 (equivalent to 10 % of total inactivation). Two peaks are visible, one at 0.2 (corresponding to I_{K2}) and one at 0.9 (corresponding to I_{K1}).



6.1.3. ACTIVATION AND DECAY

Fig. 6.3. illustrates the effect of 5 mM 4-AP, on the outward current recorded over 10 s pulses to between -50 mV and +10 mV from a holding potential of -80 mV. Fig. 6.3A and B shows currents observed between -50 and +10 mV in a cell where I_{K1} predominated, in the absence (A) and presence (B) of 5 mM 4-AP. Fig. 6.3C and D shows current in a cell where I_{K2} predominated, in the absence (C) and presence (D) of 5 mM 4-AP. I_{K1} current appeared to be slightly enhanced by 5 mM 4-AP in the example shown here, but in a group of 5 cells the difference did not reach statistical significance (7 ± 10 % enhancement at 10 s, $n = 5$). I_{K2} on the other hand, was blocked significantly by 5 mM 4-AP (63 ± 11 %, $n = 6$, $P < 0.05$).

Fig. 6.3. also illustrates two other differences between I_{K1} and I_{K2} . Firstly, I_{K1} current was apparent at -40 to -50 mV, whereas for I_{K2} current visibly activated between -40 and -30 mV. Secondly, I_{K1} decayed significantly more slowly than I_{K2} (66.2 ± 4.7 % decrease in amplitude over 10 seconds for I_{K1} vs. 82.7 ± 5.6 % over 10 seconds for I_{K2} , $P < 0.05$). To confirm this, 10 s currents were fitted with exponential curves and time constants calculated. I_{K1} decay was best fitted to a double exponential with a constant whereas I_{K2} decay was well fitted to a single exponential with a constant component. For I_{K1} at +10 mV: τ_1 was 1016 ± 316 ms and was 22 ± 4 % of total; τ_2 was 5213 ± 1068 ms and 46 ± 5 % of total, and the constant component was 32 ± 3 % of total ($n = 6$). For I_{K2} at +10 mV: τ was 2552 ± 233 ms and was 89 ± 3 % of total, and the constant component was 11 ± 3 % ($n = 6$). τ_2 was the largest component of I_{K1} and was significantly larger than the τ value of I_{K2} ($P < 0.01$). The constant component was also significantly greater for I_{K1} than for I_{K2} ($P < 0.01$). Maintaining cells with either I_{K1} or I_{K2} present at a holding potential of 0 mV for several minutes resulted in little or no net outward current, suggesting that the constant component of the 10 s currents was in fact a very slowly inactivating component. The

kinetics of activation for I_{K1} and I_{K2} were determined by fitting current traces to a first order power function:

$$y = A*(1-\exp\{-(t-K)/\tau\})^1 + B \quad \text{equ. 6.1,}$$

where A is the current amplitude (pA) at the time t (ms) and τ is the time constant. Both types of current were found to fit better to this function than to exponential or higher order power functions. Fig. 6.4. shows the fits (black traces) for representative I_{K1} and I_{K2} currents (red traces) at potentials between -40 and +10 mV and the mean time constants calculated from those fits in six and five cells, respectively, plotted against membrane potentials. As is shown in Fig. 6.4., in addition to inactivating more slowly, I_{K1} activated significantly more slowly than I_{K2} at all potentials, and displayed a noticeable delay before the exponential phase of the activation, especially at membrane potentials close to the threshold.

6.1.4. BLOCK OF I_{K1} AND I_{K2} BY TEA

The sensitivity of I_{K1} and I_{K2} to TEA was determined by investigating the effect of increasing concentrations of this agent (1, 3, 10, and 30 mM) in cells where either I_{K1} or I_{K2} predominated. As described above, I_{K1} was further isolated by defining it as the difference current which was present at a holding potential of -80, but not -40 mV, while I_{K2} was defined as the difference current which was present at a holding potential of -40, but not 0 mV. In addition, to determine the extent to which $I_{K(Ca)}$ was blocked by 1 mM TEA, a TEA concentration-response curve was constructed when the membrane potential was held for several minutes at 0 mV in order to maximise the inactivation of all voltage-gated K^+ currents. Fig. 6.5A shows the percent inhibition of I_{K1} , I_{K2} and $I_{K(Ca)}$ by TEA. I_{K1} and I_{K2} demonstrated partial block by TEA. At 1 mM, both I_{K1} and I_{K2} were significantly inhibited (by $24 \pm 8 \%$ and $29 \pm 5 \%$ respectively $n = 5-6$), in contrast to $I_{K(Ca)}$ which was

inhibited to a much greater extent ($75 \pm 3 \%$, $n = 4$, $P < 0.01$) compared to both I_{K1} and I_{K2} .

The I - V relationships for $I_{K(Ca)}$ in the absence and presence of 1 mM TEA (in 1.5 mM Ca^{2+} PSS and without Cd^{2+}) are described in Fig. 6.5. The I - V curve, as well as the lack of outward current at the holding potential of 0 mV, suggested that $I_{K(Ca)}$ activated positive of 0 mV. There was a small but insignificant additional inhibition of this current when in addition to the application of 1 mM TEA, Ca^{2+} was removed and 0.5 mM $CdCl_2$ was present ($74 \pm 5 \%$ in PSS $n = 4$ vs. $80 \pm 6 \%$ in Ca^{2+} free PSS with 0.5 mM $CdCl_2$, $n = 4$).

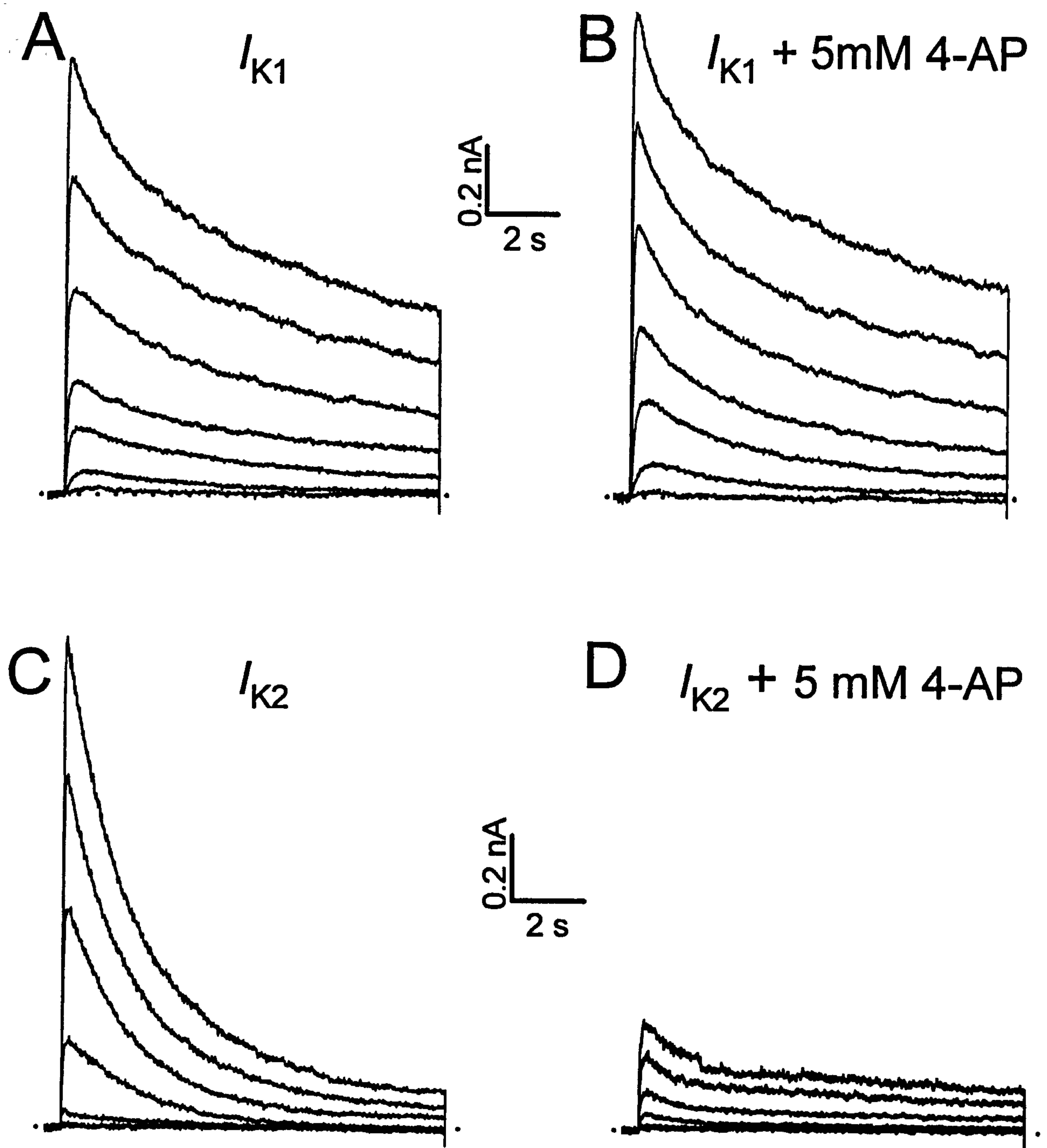
The blockade of I_{K1} by 1 mM TEA was also measured in the presence of 1 μ M paxilline, defining I_{K1} as the difference current present at a holding potential of -80 but not -40 mV in cells where this current predominated. Under these conditions, 1 mM TEA inhibited the current by $27 \pm 1 \%$, ($n = 5$). This value was similar to that recorded in the absence of paxilline.

These experiments suggested that 1 mM TEA caused a small but significant inhibition of I_{K1} . To determine whether this partial block by TEA or whether the removal of Ca^{2+} and addition of Cd^{2+} were affecting the voltage-dependent characteristics of I_{K1} , availability experiments were repeated in Ca^{2+} free PSS containing 1 μ M paxilline, an alternative more selective blocker of $I_{K(Ca)}$, as well as 5 mM 4-AP and 0.5 mM $CdCl_2$. Availability experiments were also carried out in the presence of normal- Ca^{2+} PSS containing 10 mM TEA and 5 mM 4-AP. 1 μ M paxilline was found to cause inhibition of $I_{K(Ca)}$ comparable to that caused by 10 mM TEA (using a 0 mV holding potential, data not shown). The inactivation of I_{K1} under both sets of conditions was similar to that described in Fig. 6.1. Half-inactivation potentials and the proportion of non-inactivating current under all three sets of conditions are summarised in Table 6.1. Using ANOVA for multiple comparisons, there were no significant differences in any of the three parameters between the four sets

of conditions, although differences in the size of the non-inactivating component came close to statistical significance ($P = 0.065$).

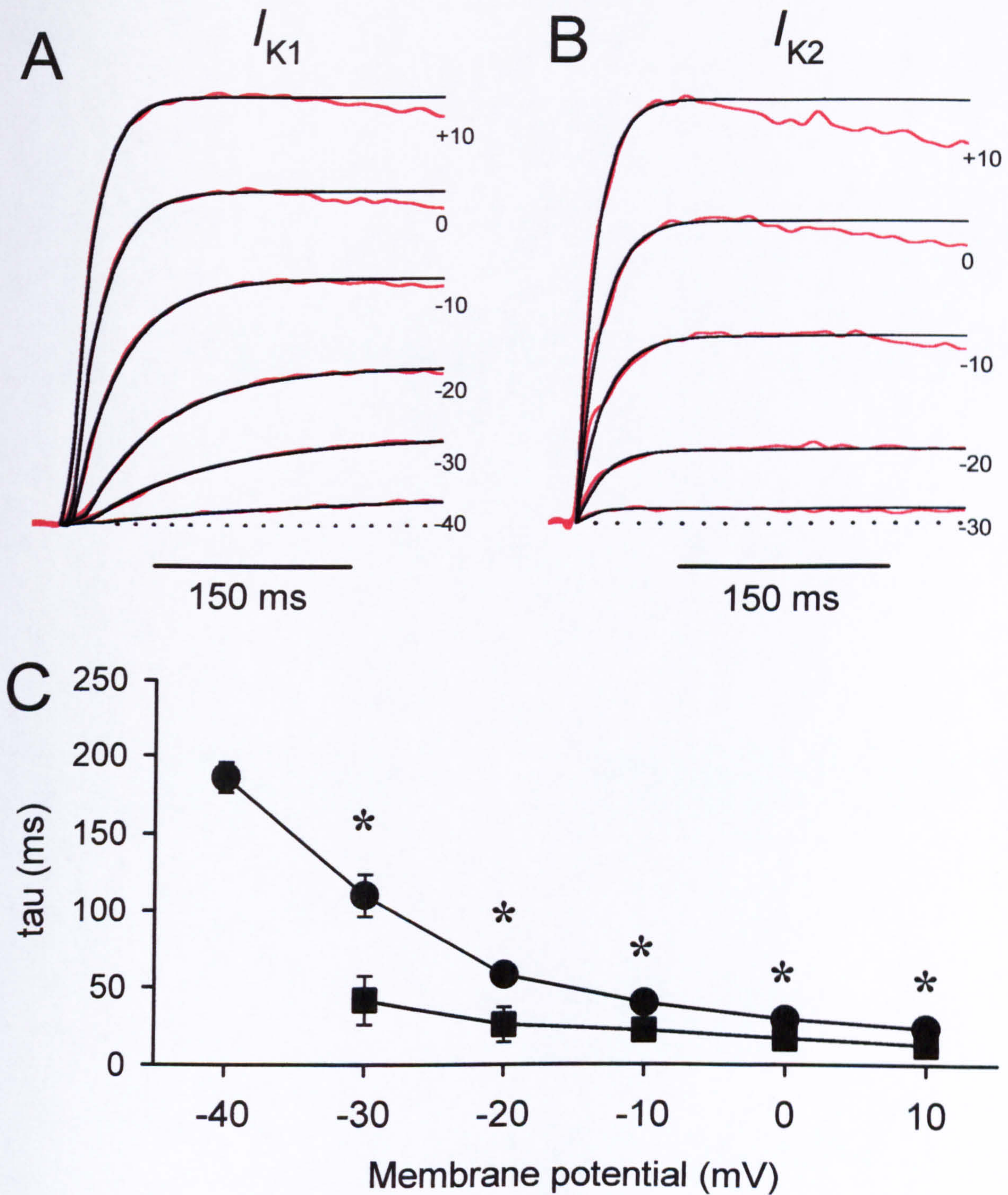
In two additional pregnant HMSMCs, one with 10 mM TEA and 5 mM 4-AP already in the bath and the other with 10 mM TEA, 5 mM 4-AP and 100 μ M clofilium (see below) already in the bath, 20 μ M niflumic acid was applied, in order to determine the contribution, if any, of chloride current to the outward current at +60 mV from an V_H of -80 mV. At this concentration, niflumic acid did not inhibit the remaining current. Indeed in the first cell the current was in fact increased by $\sim 20\%$.

Fig 6.3. Decay and 4-AP sensitivity of I_{K1} and I_{K2}



10 second depolarisations to between -50 and +10 mV from a holding potential of -80 mV for I_{K1} (A and B) and I_{K2} (C and D). A: I_{K1} in the absence of 4-AP. B: I_{K1} in the presence of 5 mM 4-AP in the same cell as in A. C: I_{K2} in the absence of 4-AP. D: I_{K2} in the presence of 5 mM 4-AP in the same cell as in C. The dotted lines represent zero current.

Fig 6.4. Activation kinetics of I_{K1} and I_{K2}



A and B: Currents elicited by depolarisations to between -40 and +10 mV (red traces) in cells where I_{K1} (**A**) and I_{K2} (**B**) predominate with fitted curves (solid black lines) superimposed (see text for fitting procedure). The dotted lines represent zero current.

C: a plot of time constants (τ) against membrane potential (mean and S.E.M.) (I_{K1} = ● I_{K2} = ■). Asterisks represent significant differences at $P < 0.05$.

FIGURE 6.5. EFFECT OF TEA ON I_{K1} , I_{K2} AND $I_{K(Ca)}$

A: Dose response for TEA, plotting the mean \pm SEM % inhibition of I_{K1} (\square , $n = 5-6$), I_{K2} (\blacksquare , $n = 5-6$) and $I_{K(Ca)}$ (\bullet , $n = 4$) against TEA concentration. All three currents were significantly inhibited by all concentrations of TEA ($P < 0.05$). B: I - V relationship for $I_{K(Ca)}$ in 4 paired cells in the absence (\bullet) and presence (\circ) of 1 mM TEA, plotting the mean \pm S.E.M. of current (normalised to the current at +60 mV in the absence of TEA) against membrane potential. I_{K1} and I_{K2} were recorded using the 3 step inactivation protocol (see text). $I_{K(Ca)}$ was recorded in normal PSS (1.5 mM Ca^{2+}) from a holding potential of 0 mV and a test potential of +60 mV.

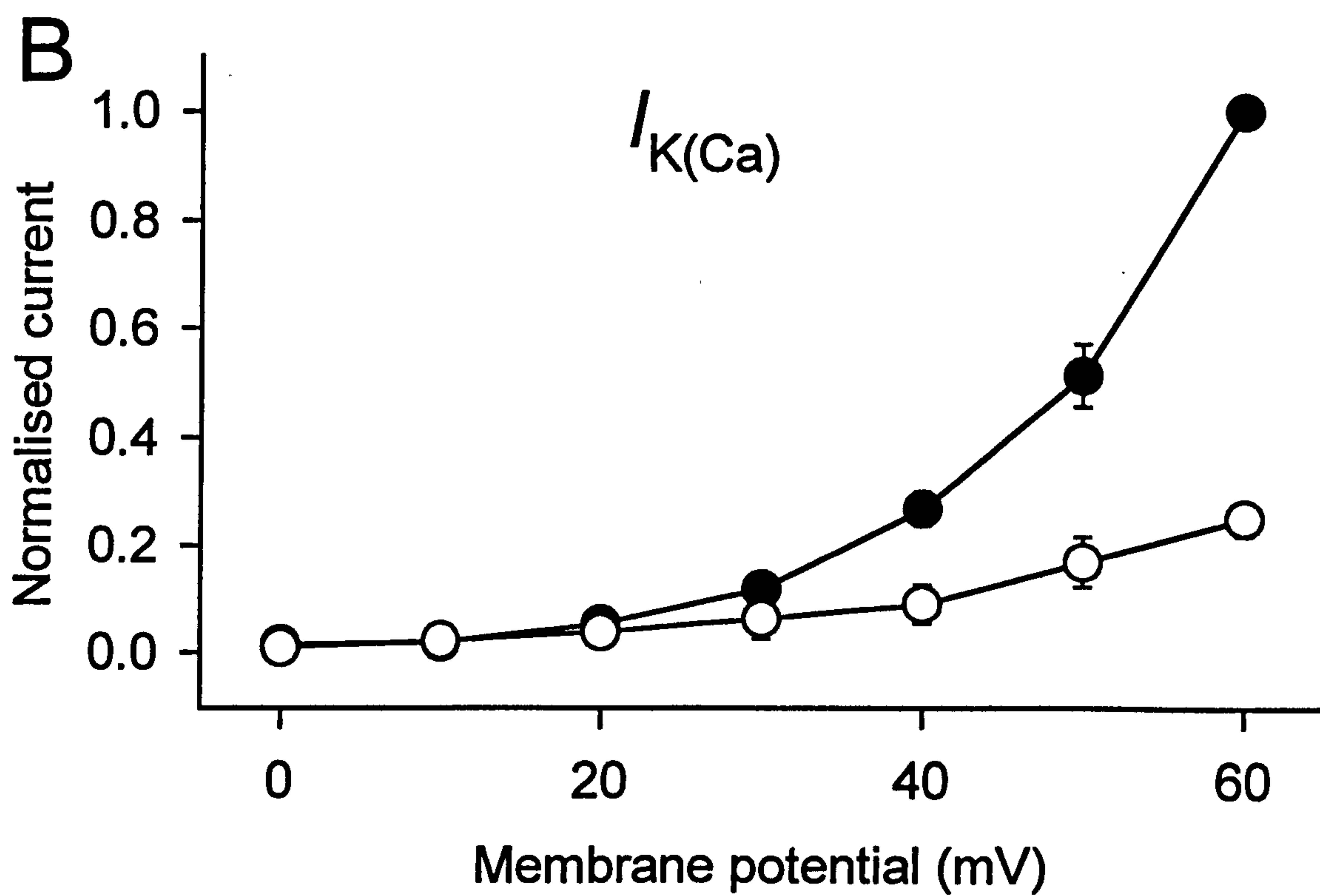
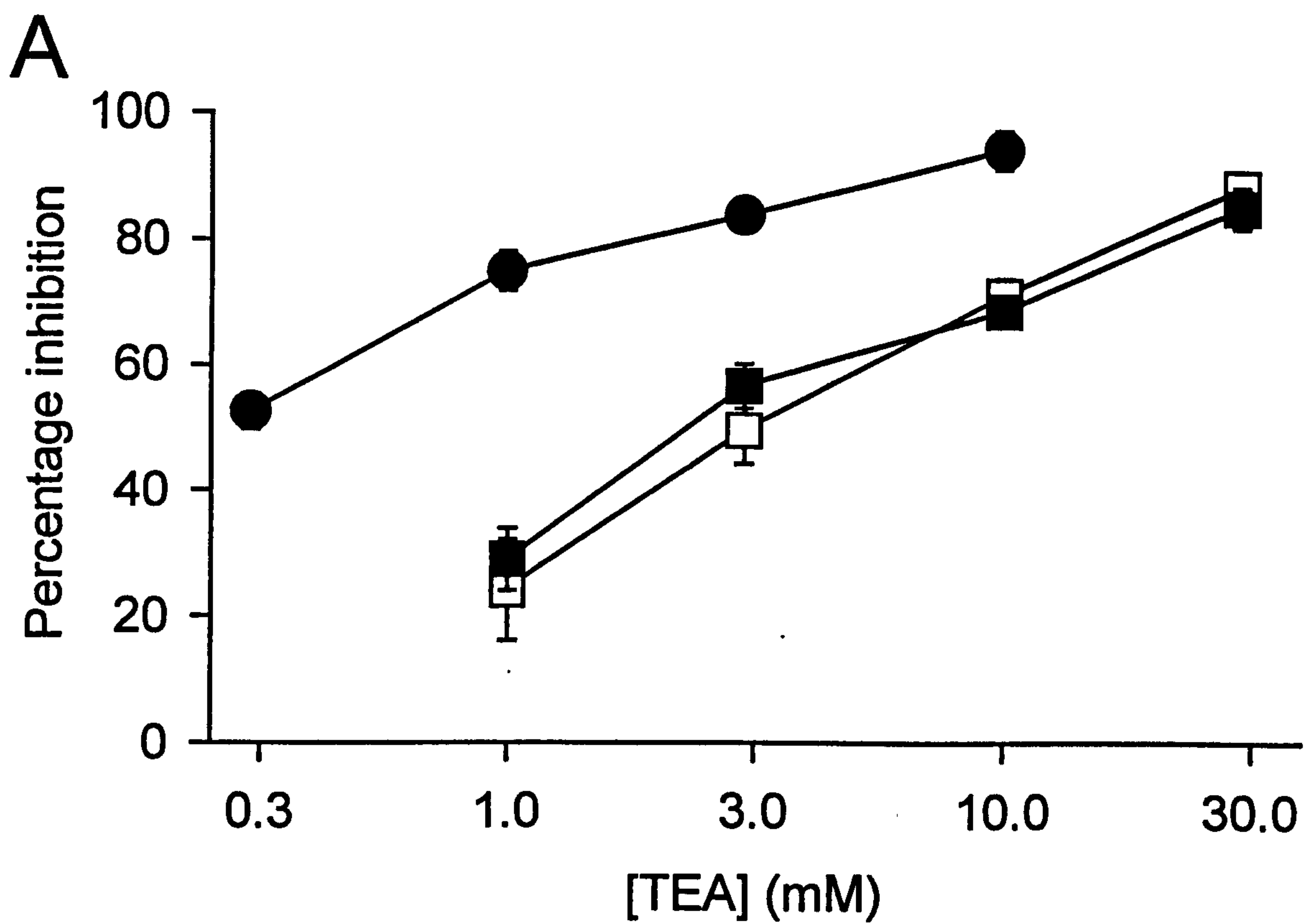


TABLE 6.1. EFFECT OF BATH SOLUTION ON I_{K1} INACTIVATION CHARACTERISTICS.

Bath Solution	$V_{0.5}$	k	Non-inactivating component (%)
1 μ M paxilline, 0.5 mM CdCl_2 , 5 mM 4-AP	$-61 \pm 3 \text{ mV}$ (n = 5)	$8 \pm 0.4 \text{ mV}$ (n = 5)	$43 \pm 3 \%$ (n = 5)
1 mM TEA, 0.5 mM CdCl_2	$-65 \pm 3 \text{ mV}$ (n = 6)	$8 \pm 2 \text{ mV}$ (n = 6)	$36 \pm 2 \%$ (n = 6)
1 mM TEA, 0.5 mM CdCl_2 , 5 mM 4-AP	$-66 \pm 2 \text{ mV}$ (n = 6)	$9 \pm 4 \text{ mV}$ (n = 6)	$36 \pm 1 \%$ (n = 6)
10 mM TEA, 1.5 mM CaCl_2 , 5 mM 4-AP	$-67 \pm 3 \text{ mV}$ (n = 5)	$8 \pm 2 \text{ mV}$ (n = 5)	$30 \pm 5 \%$ (n = 5)

Half-inactivation potentials ($V_{0.5}$), slope factor (k) and percentage of I_{K1} at +60 mV which does not inactivate, calculated by fitting steady-state inactivation data to Boltzmann distribution. There were no significant differences in any of the three variables between the four sets of conditions (using ANOVA for multiple comparisons).

6.1.5. VOLTAGE-DEPENDENCE OF ACTIVATION OF I_{K1}

The threshold of activation of I_{K1} when paxilline was used to block $I_{K(Ca)}$ was also similar to that seen with 1 mM TEA. To investigate this further, the I - V relationship of I_{K1} was constructed using depolarisations to between -80 mV and +60 mV from a holding potential of -80 mV, in a Ca^{2+} free bath solution containing 1 μ M paxilline, 0.5 mM $CdCl_2$ and 5 mM 4-AP. Fig. 6.6. shows example traces (A), the mean normalised I - V relationship (B) and the normalised current activation relationship calculated from this data (C, $n = 5$). The activation relationship ($\frac{I}{I_{\infty}}$ chord conductance) was calculated from the equation $y = I/(V_m - E_r)$ where I is current amplitude, V_m is the membrane potential and E_r is the reversal potential (calculated as -83 mV). These data were then fitted to the Boltzmann equation and the mean half activation potential and slope factor in the 5 cells were calculated as $+1.2 \pm 4.4$ mV (indicated by dotted black line) and -16 ± 1 mV, respectively (Fig 6.6C). The mean normalised availability curve and mean half inactivation potential for I_{K1} with 1 μ M paxilline, 0.5 mM $CdCl_2$ and 5 mM 4-AP in the bath solution (red lines) are also shown in Fig 6.6C, super-imposed on the normalised activation curve. The solid red line shows inactivation inclusive of the non-inactivating component of I_{K1} . Alternatively, taking into account the possibility that only a part of the non-inactivating component is I_{K1} , the inactivation curve with the non-inactivating component removed is also shown (dashed red line). This second curve bisects the activation curve at a membrane potential where the current is not fully inactivated, implying a window current (at membrane potentials at which activation and inactivation are balanced in favour of activation a steady-state current may result) for I_{K1} between -60 mV and -20 mV, with a peak at about -36 mV.

6.1.6. EFFECTS OF 4-AP AND CLOFILIUM ON I_{K1} AND I_{K2}

The effects of increasing concentrations of 4-AP (0, 1, 2, 5, 10, and 20 mM) on I_{K1} and I_{K2} were determined using the 3-step inactivation protocol described above (prepulses to -80, -40 and 0 mV), and are shown in Fig. 6.7A and B, respectively. In Fig. 6.7C and D, the

relative inhibition (or enhancement) by each concentration of 4-AP on I_{K1} and on I_{K2} components respectively, are plotted. I_{K1} was significantly enhanced by 10 and 20 mM 4-AP ($P < 0.05$, $n = 5$), whereas I_{K2} was significantly inhibited by all concentrations of 4-AP tested ($P < 0.001$ for each concentration, $n = 5$).

The effects of several concentrations of clofilium (a blocker of voltage-gated K^+ currents) on I_{K1} and a single concentration (100 μ M) on I_{K2} , in representative cells are shown in Figs. 6.8A & B, respectively. Currents were elicited by 10 second pulses to 0 mV from a holding potential of -80 mV in Ca^{2+} free PSS containing 0.5 mM $CdCl_2$ and 1 mM TEA. The predominance of either I_{K1} or I_{K2} was confirmed in each cell with the 3-step inactivation protocol. Decay of I_{K1} was enhanced in a concentration-dependent manner by clofilium such that at 100 μ M, the current was nearly abolished after 10 s. Percent block of I_{K1} at peak and end of pulse are presented in Fig. 6.8C ($n = 5-8$). In cells where I_{K2} was the predominant current (as in Fig 6.8B, also defined using the 3-step inactivation protocol), I_{K2} was also profoundly inhibited by 100 μ M clofilium (90 ± 5 % at 200-300 ms; 96 ± 3 % at 10 s, $n = 4$). The effect of 100 μ M clofilium on I_{K1} was also evaluated over a range of membrane potentials between -80 mV and +60 mV in 4 cells, with normal Ca^{2+} -PSS containing 10 mM TEA and 5 mM 4-AP in the bath solution. The resultant mean normalised $I-V$ curve is presented in Fig 6.8D. Clofilium was less potent at potentials positive to 0 mV, however this was only significant at +60 mV (90 ± 3 % block at 0 mV vs. 68 ± 6 % at +60 mV, $P < 0.05$). The non-inactivating component of I_{K1} , defined as the fraction of the current at +60 mV not inactivated following a 10 s conditioning pulse to 0 mV, was also less sensitive to 100 μ M clofilium (39 ± 10 % block, $n = 6$, $P < 0.05$).

FIG 6.6. VOLTAGE-DEPENDENCE OF ACTIVATION OF I_{K1}

A: I_{K1} Currents elicited by 300 ms depolarisations to between -80 and +60 mV from a holding potential of -80 mV, using 1 μ M paxilline, 0.5 mM CdCl_2 and 5 mM 4-AP in a Ca^{2+} free bath solution. **B:** Plot of current measured at the end of the 300 ms pulse against membrane potential, normalised to the current at +60 mV (■, mean \pm S.E.M., $n = 5$). **C:** Activation relationship of I_{K1} plotting current, converted to conductance by the equation $y = I/(V_m - E_r)$ (see text) and normalised to the +60 mV values, against membrane potential (●, mean \pm S.E.M., $n = 5$). The mean inactivation curve derived for I_{K1} using the same bath conditions (see table 6.1.) is also plotted, with (solid red line) and without (dashed red line) the “non-inactivating component”. The dotted black and red lines represents the mean half activation and inactivation potentials, respectively.

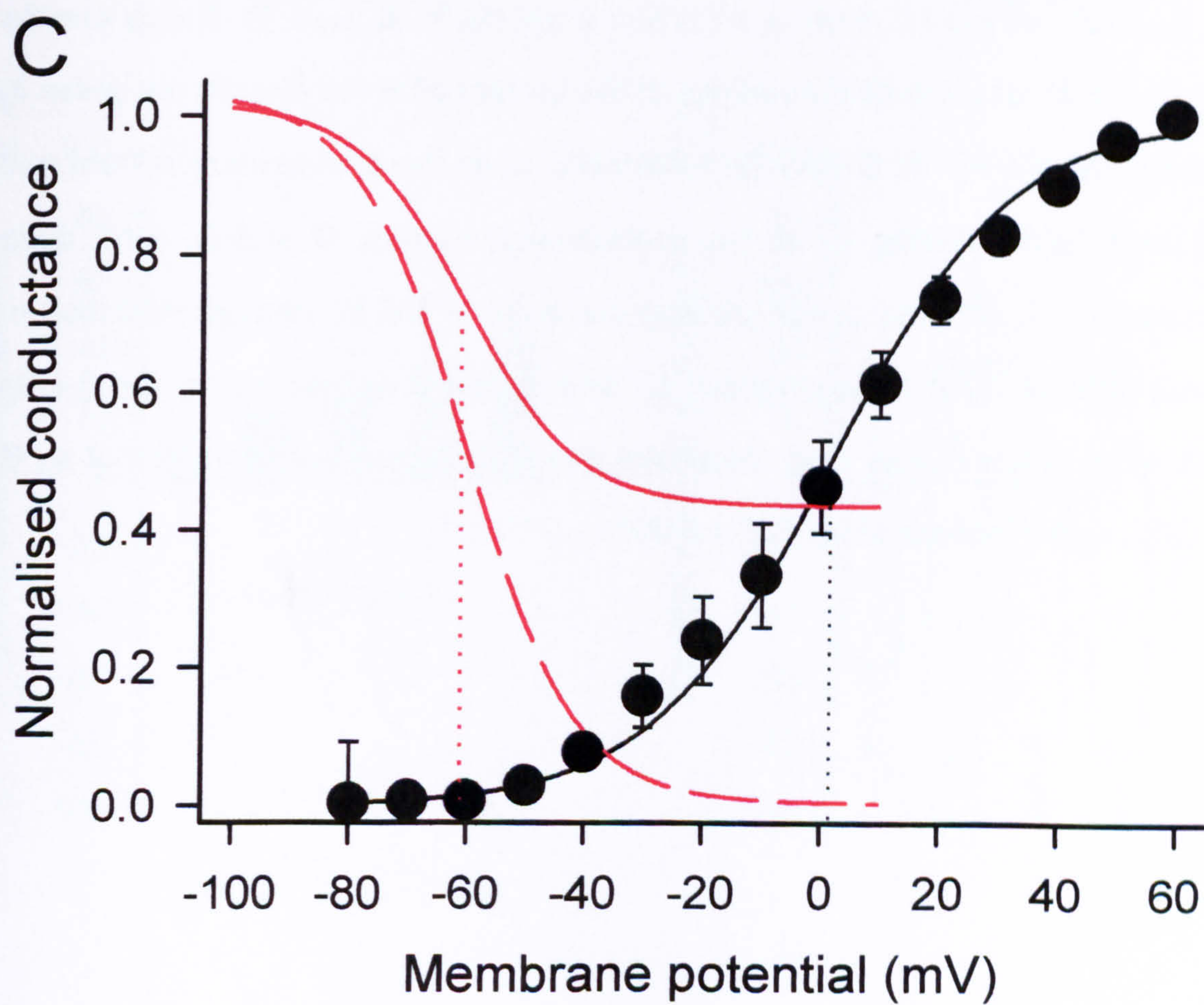
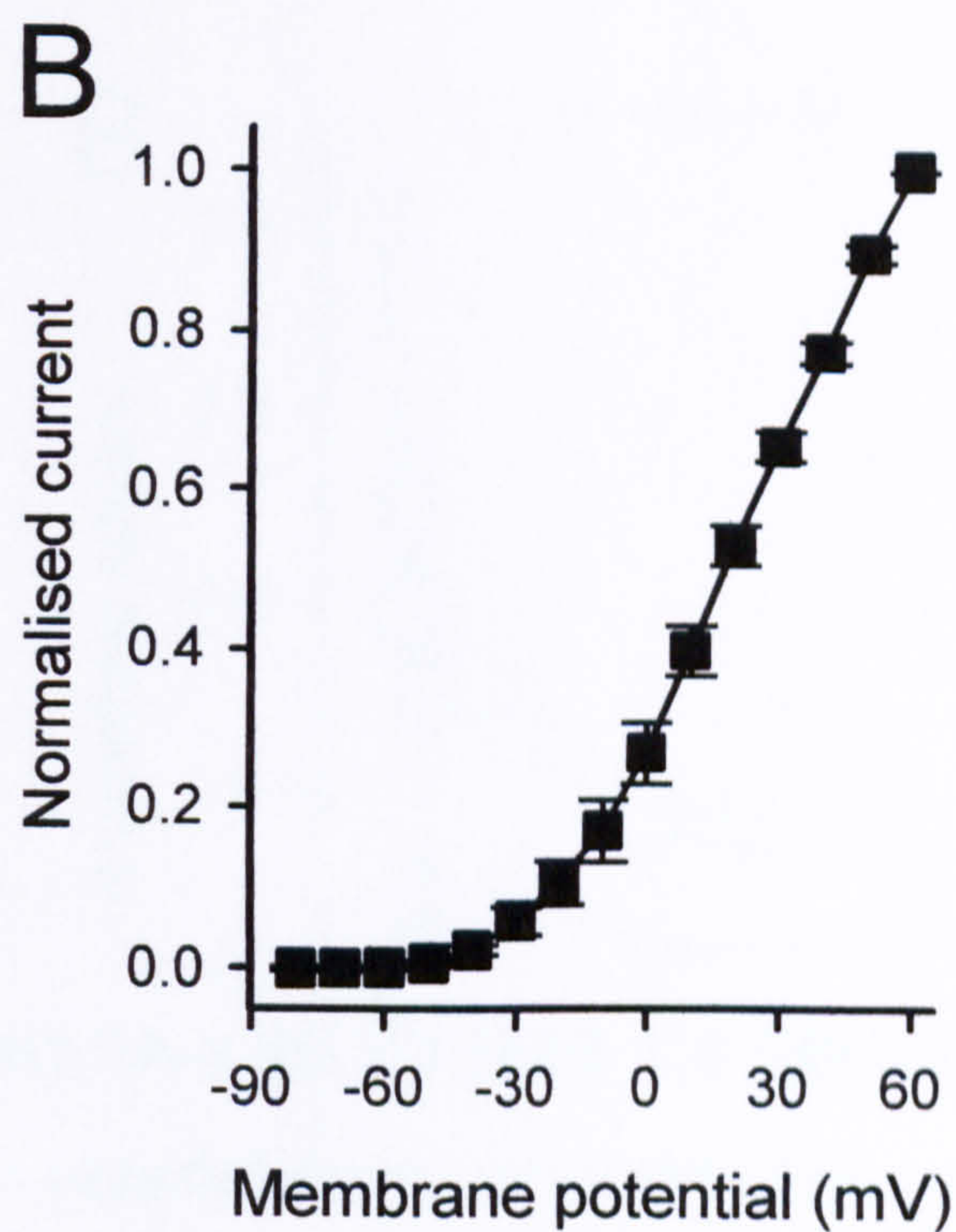
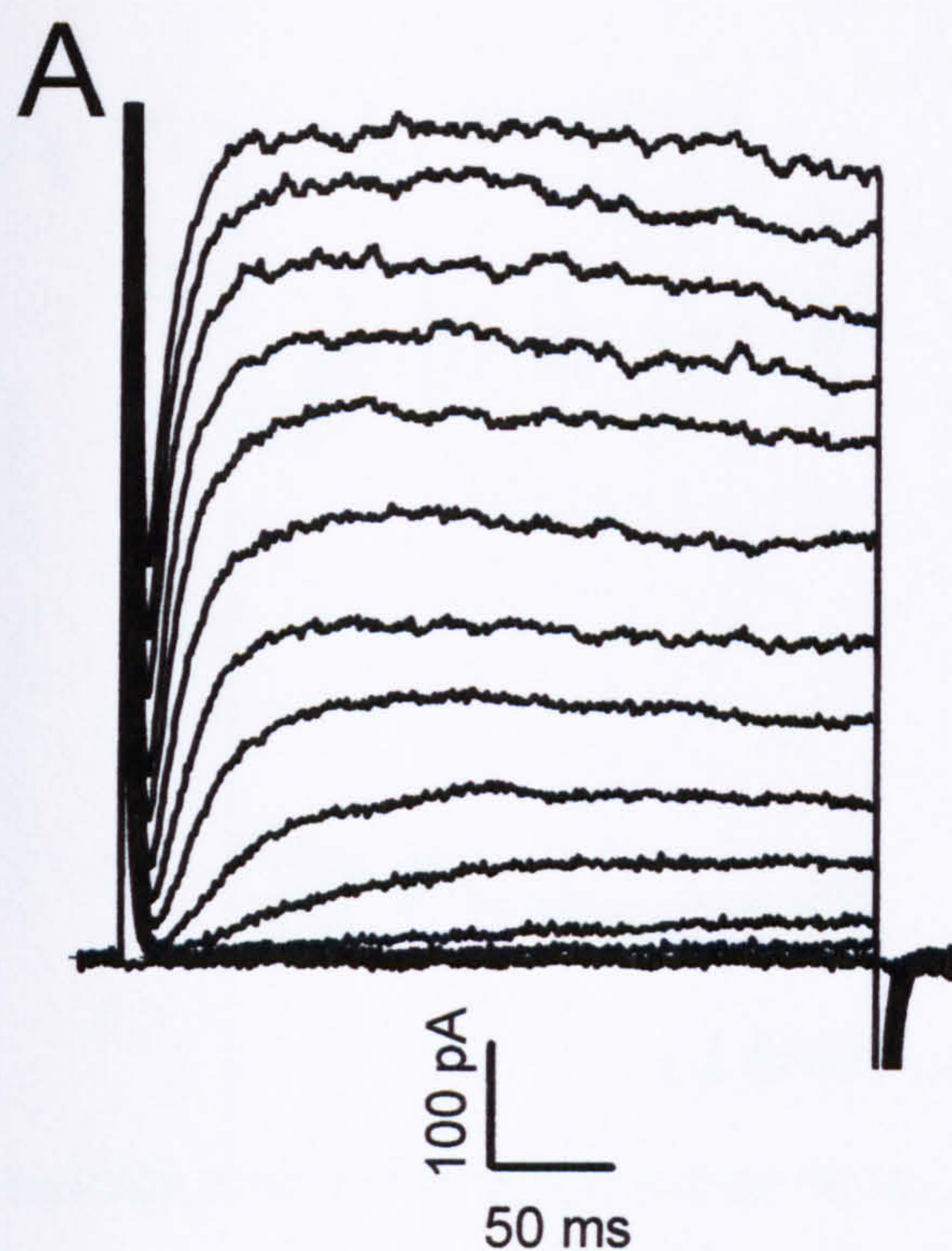


FIGURE 6.7. EFFECT OF 4-AP ON I_{K1} AND I_{K2}

(A and B): Plots of 4-AP concentration against measurements of currents elicited by +60 mV test pulses following 10 second conditioning pre-pulses to -80 mV (I_{K1} = ■, I_{K2} = □), -40 mV (I_{K1} = ●, I_{K2} = ○) and 0 mV (I_{K1} = ▲, I_{K2} = ▽). Points are the mean \pm S.E.M. of currents normalised to the current after the -80 mV pre-pulse in the absence of 4-AP. A: 5 cells demonstrating I_{K1} as the predominant current and B: 5 cells demonstrating I_{K2} as the predominant current. C and D: 4-AP dose response for I_{K1} (C) and I_{K2} (D) plotting the mean \pm S.E.M. percent inhibition of current against 4-AP concentration. I_{K1} and I_{K2} were defined using the 3-step inactivation protocol (see text). Asterisks show significant enhancement of I_{K1} (* $P < 0.05$) and inhibition of I_{K2} (** $P < 0.001$).

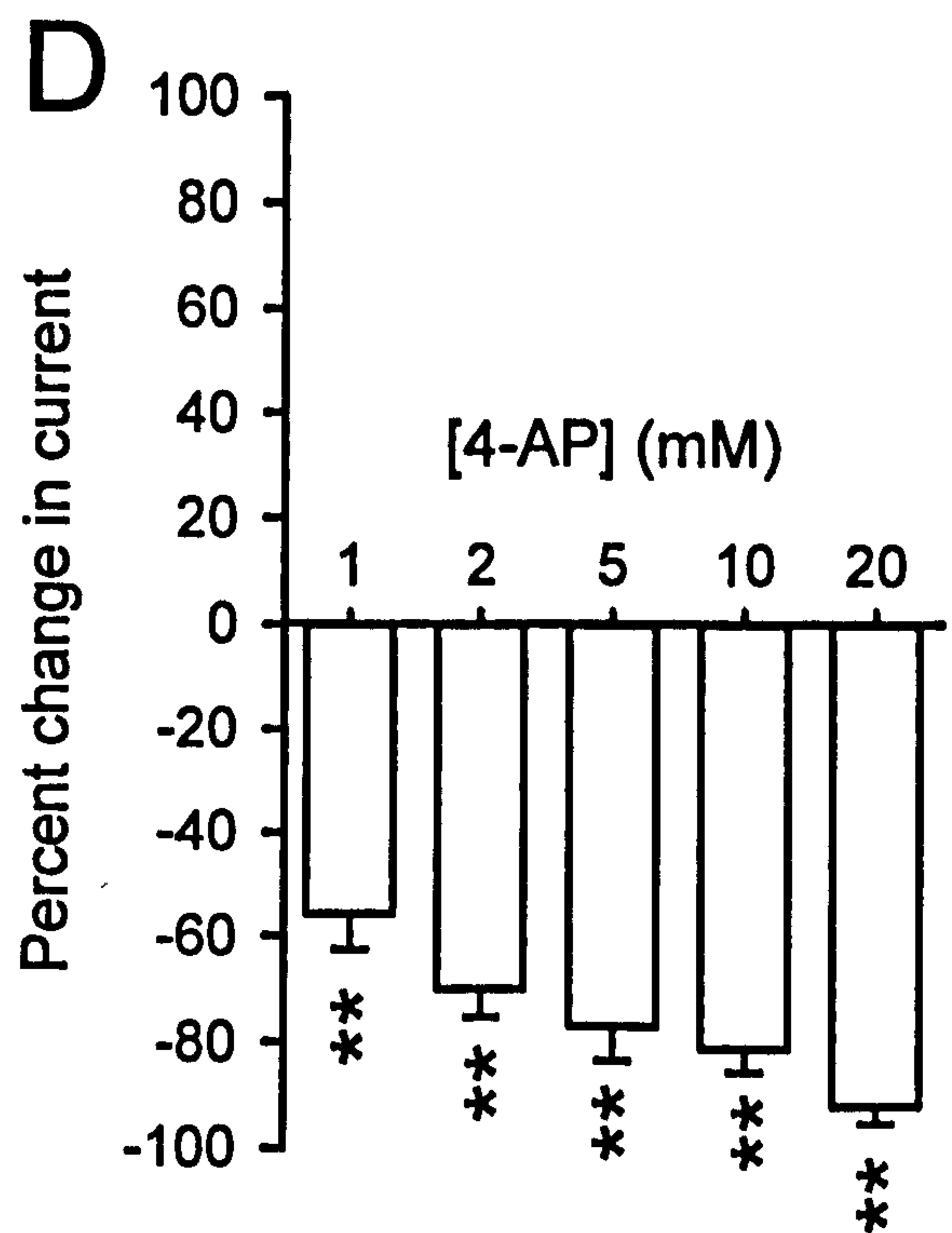
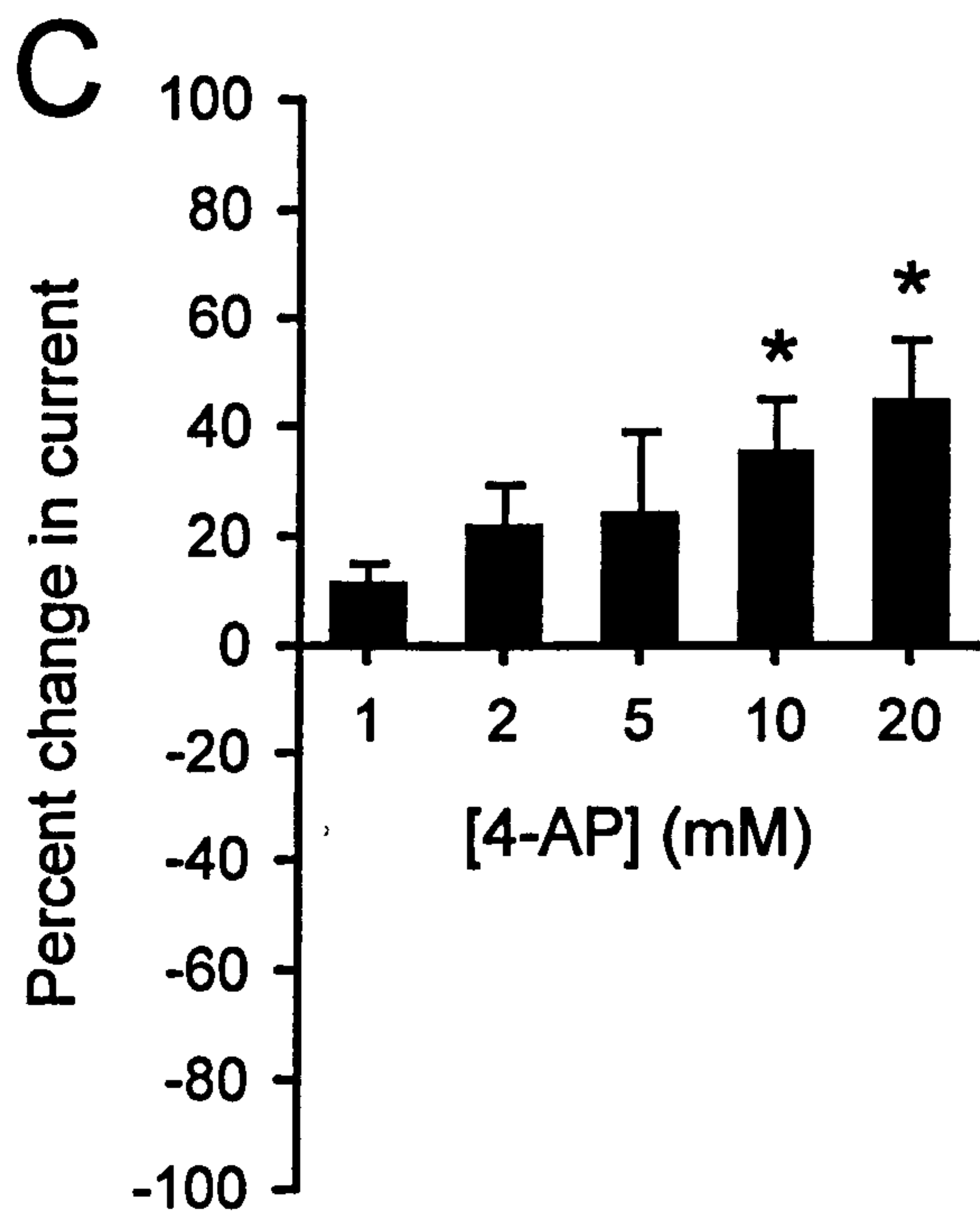
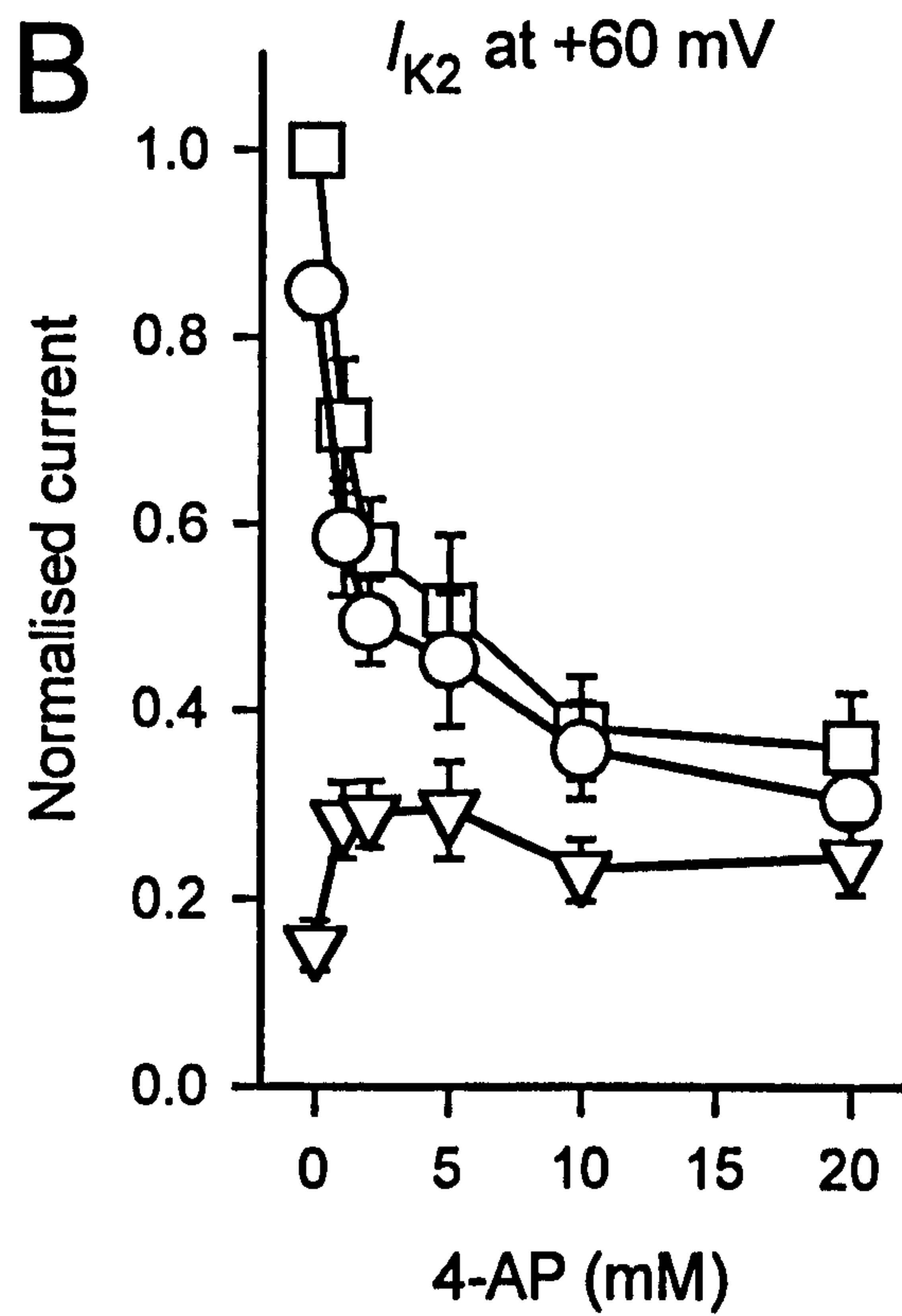
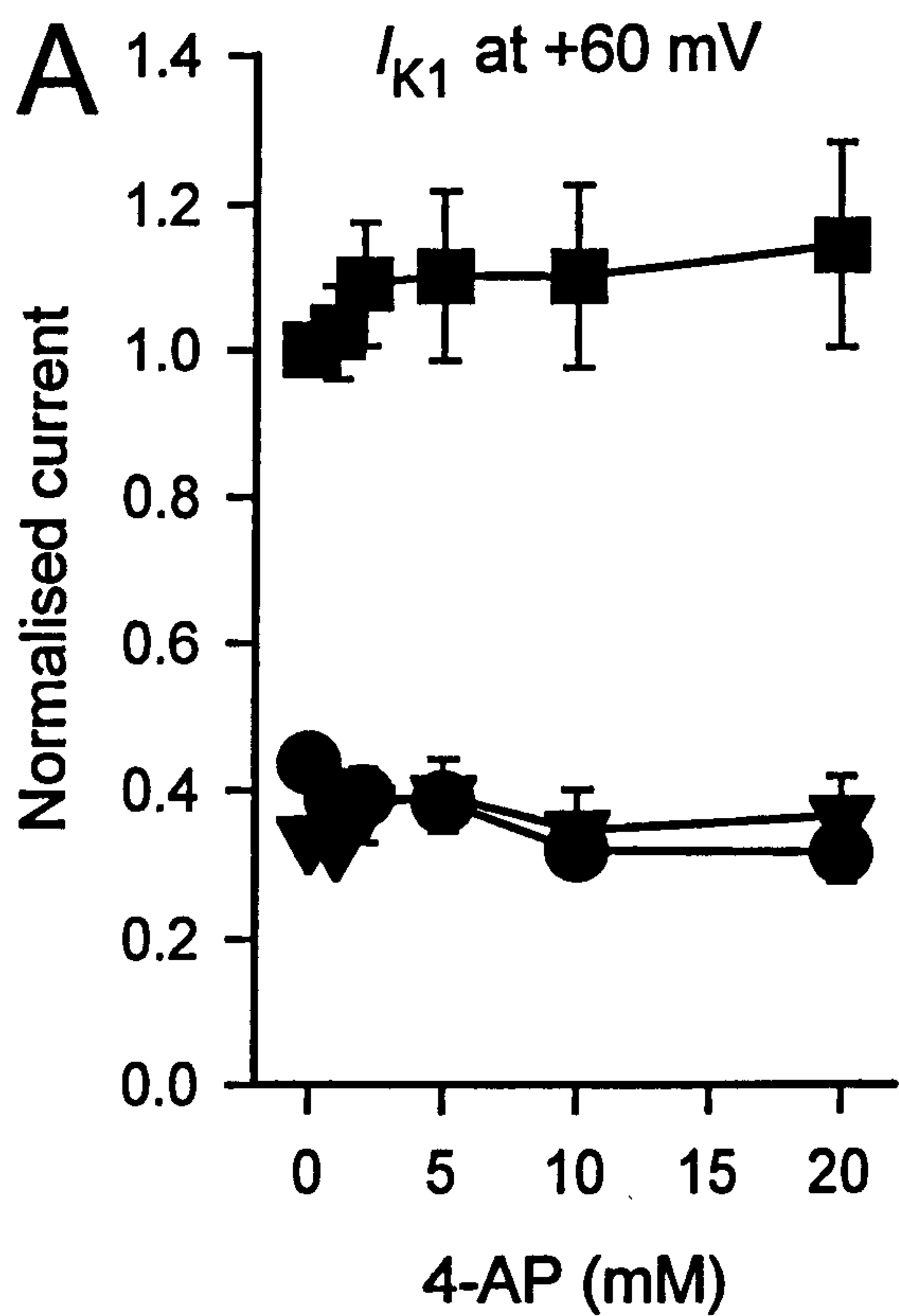
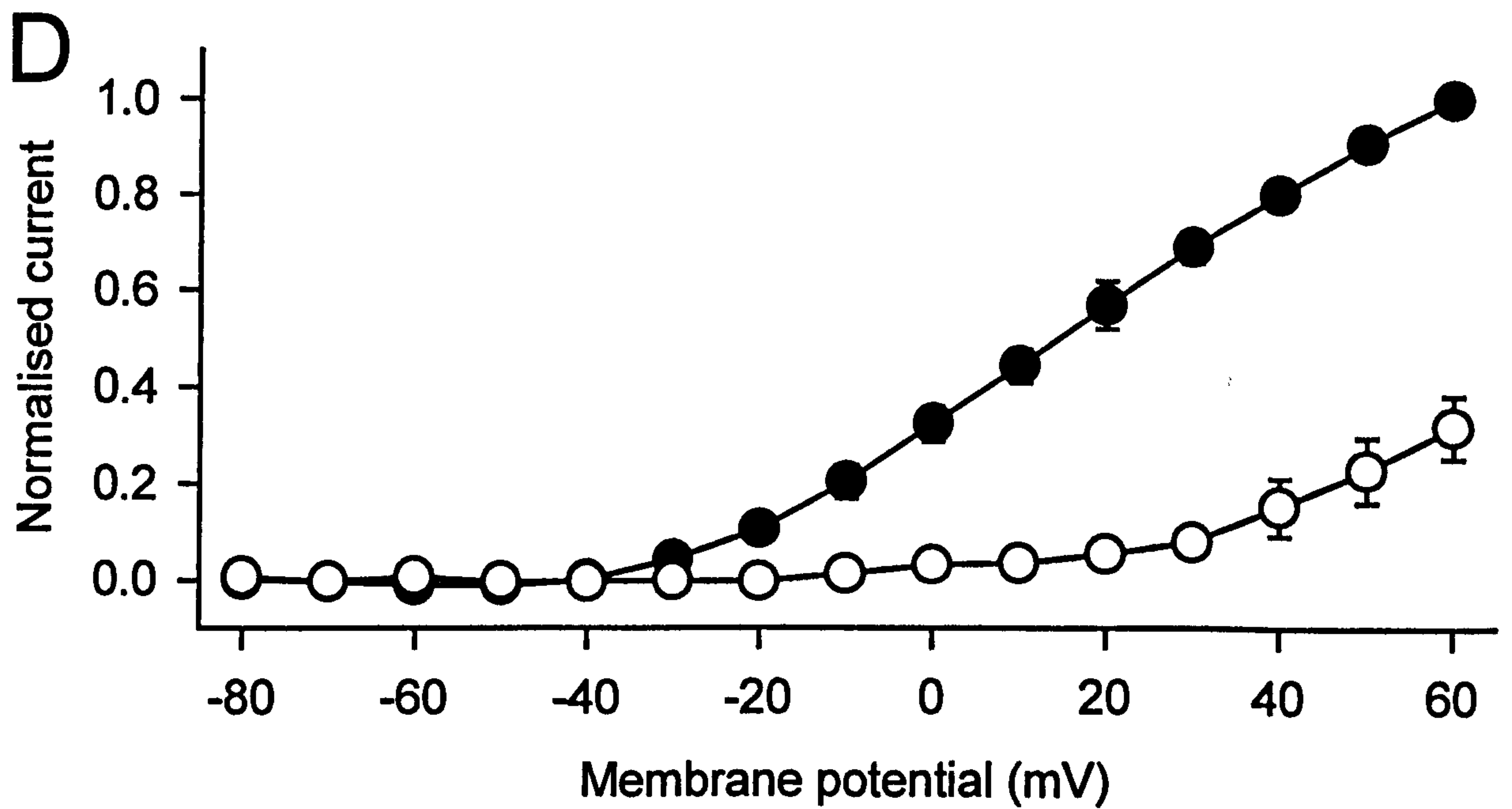
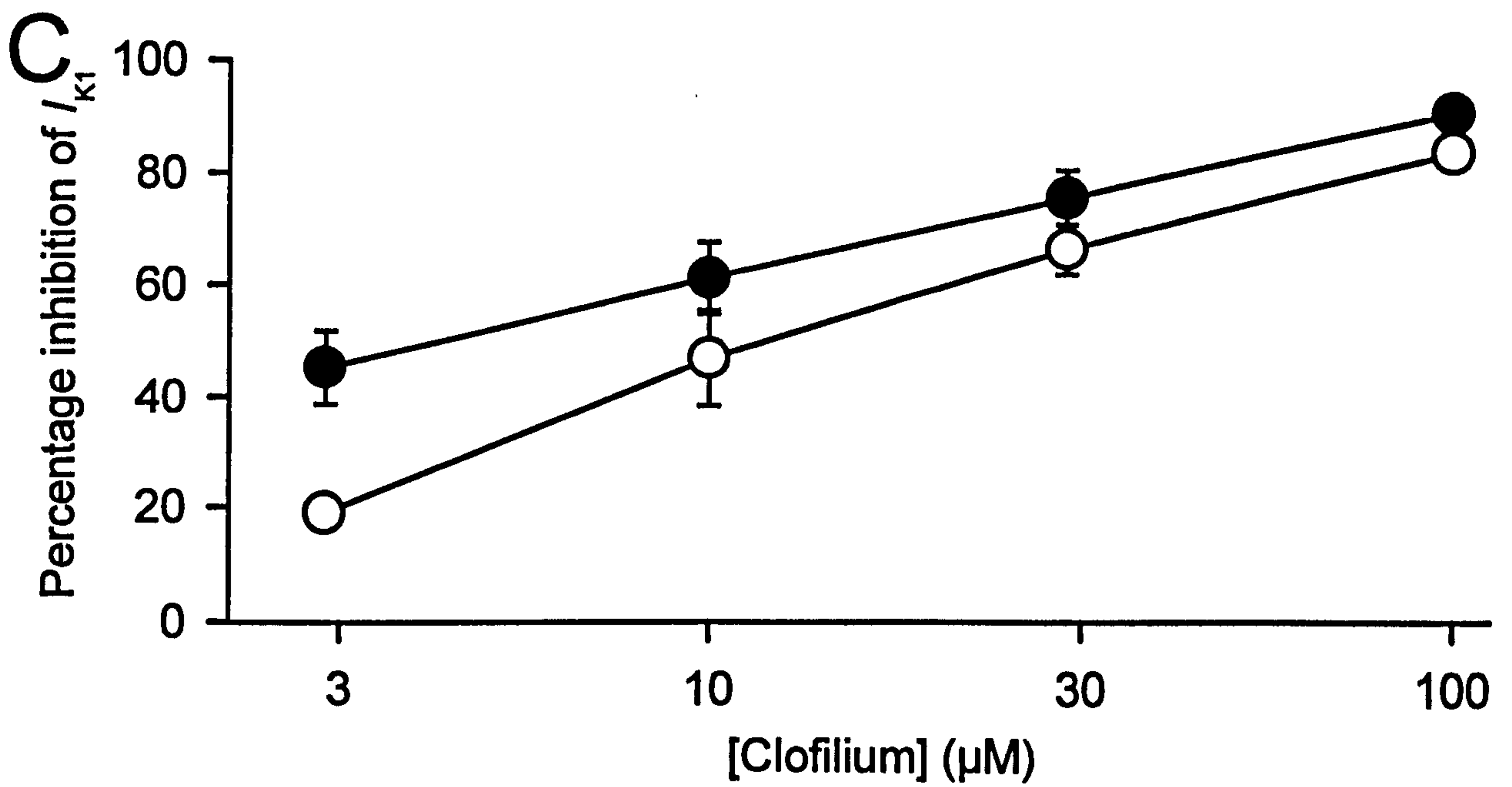
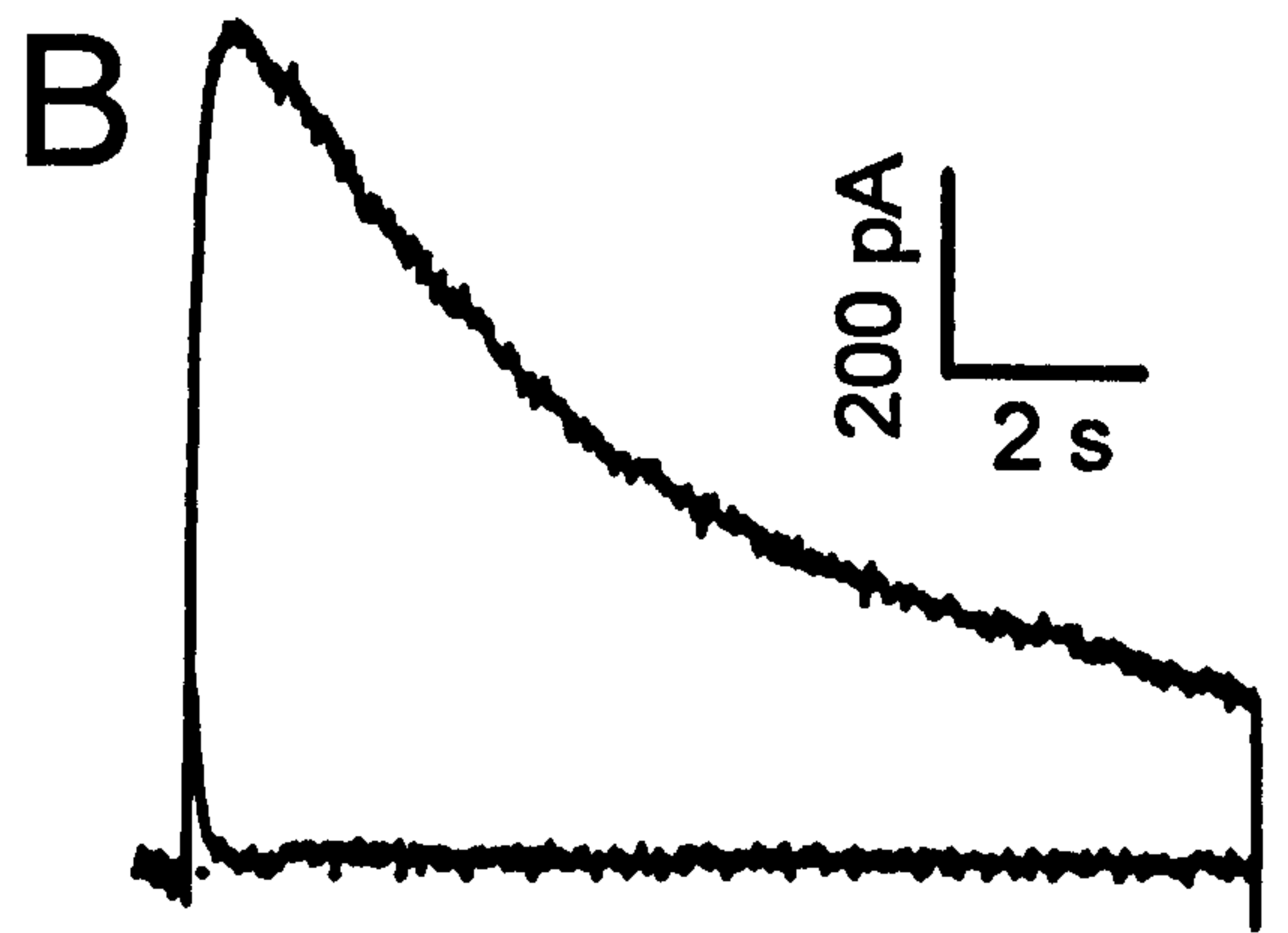
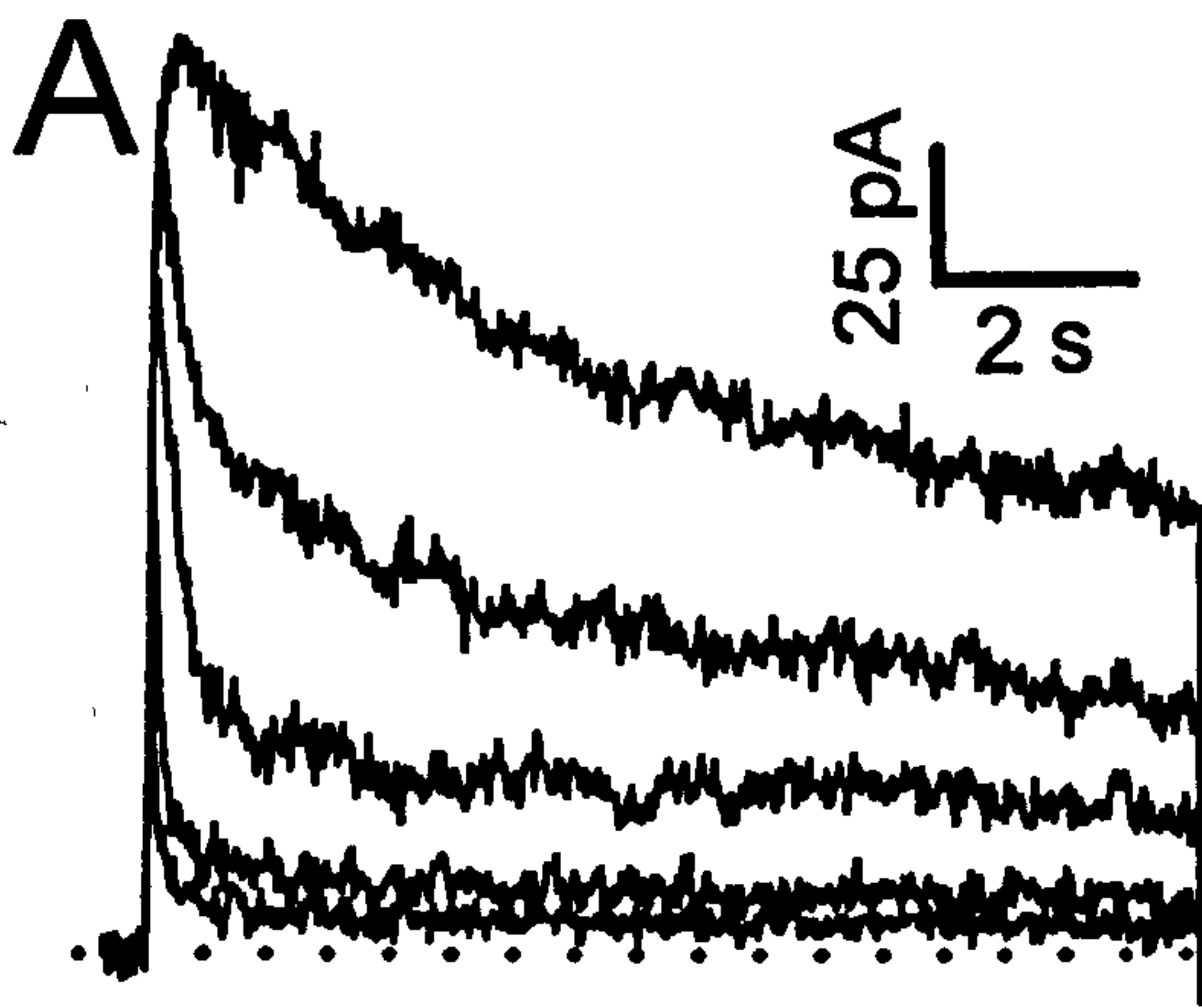


FIGURE 6.8. EFFECT OF CLOFILIUM ON I_{K1} AND I_{K2}

A: Currents elicited by 10 second pulses to 0 mV from a holding potential of -80 mV, in a cell where I_{K1} predominated in the absence and presence of 3, 10, 30 and 100 μ M clofilium. The dotted lines represent zero current. **B:** Effect of 100 μ M clofilium on I_{K2} under identical conditions as for I_{K1} in **A**. **C:** Dose response for the effect of clofilium on I_{K1} , plotting the mean and S.E.M. ($n = 5-7$) percentage inhibition measured at 200-300 ms (●) and 10 s (○) against clofilium concentration. **D:** I-V relationship for I_{K1} with 10 mM TEA and 5 mM 4-AP in Ca^{2+} -PSS bath solution in the absence (●) and presence (○) of 100 μ M clofilium, plotting mean \pm S.E.M. current normalised to the control at +60 mV ($n = 4$).



6.2. DISCUSSION

As suggested in chapter 5, the delayed rectifier K^+ current in HMSMCs from pregnant women possesses two distinct components (I_{K1} and I_{K2}). Here, the characteristics of I_{K1} and I_{K2} and their similarity with other native K^+ currents in myometrium and other contractile tissues and with cloned K_v channels is discussed. The possible physiological function of I_{K1} in the myometrium is also evaluated.

6.2.1. ISOLATION OF I_{K1} AND I_{K2}

The isolation of I_{K1} and I_{K2} required the elimination of the other currents present in these cells. $I_{K(Ca)}$ was routinely minimised firstly by excluding Ca^{2+} from, and adding 0.5 mM Cd^{2+} to, the bath solution. This had the additional benefits of blocking the voltage-gated Ca^{2+} current, and diminishing $I_{K,A}$ (see chapter 5), since no transient 4-AP sensitive current was present under these conditions. The three parameters of the voltage-dependence of inactivation of I_{K1} ($V_{0.5}$, k and non-inactivating component) were not altered by the replacement of 1.5 mM Ca^{2+} with 0.5 mM Cd^{2+} in the bath solution. Secondly, either 1 mM TEA or 1 μ M of the selective antagonist paxilline (Sanchez & McManus, 1996) was utilised to block $I_{K(Ca)}$. Both combinations of conditions reduced the outward current measured at +60 mV from a holding potential of 0 mV, by about 80 %. The inactivation of the voltage-gated currents at this holding potential, combined with the concentration-dependence of the block of this current by TEA (Fig. 6.5.), suggest strongly that the current inhibited under these conditions was almost entirely $I_{K(Ca)}$. $I_{K(Ca)}$ activated only at potentials positive of 0 mV under the conditions used in this study but comprised a substantial component of the whole cell current at more positive potentials. It was inhibited by TEA with an approximate EC_{50} of 0.3 mM.

In most experiments it was also required that I_{K1} and I_{K2} be isolated from each other. This was accomplished using several approaches. I_{K1} was always studied in cells where it was the predominant current, as confirmed using the 3-step inactivation protocol described above. In some of those cells (*eg* results shown in Figures 6.4 and 6.8.), I_{K2} contributed such a small component of current that it was ignored. In others the I_{K2} component, was blocked using 5 mM 4-AP (*eg* results shown in Fig. 6.6. and Table 6.1). Finally, in the remaining experiments (results shown in Figures 6.5. and 6.7.) I_{K1} was defined as the difference between the currents recorded at +60 mV after the cell was subjected to conditioning potentials at -40 and -80 mV. Similarly, I_{K2} was always investigated in cells in which it predominated. In cases where this predominance was not extreme, it was defined as the difference between the currents recorded at +60 mV after the cell was subjected to conditioning potentials at -40 and 0 mV (Figures 6.5. and 6.7.).

6.2.2. CHARACTERISTICS OF I_{K1} ; COMPARISONS WITH OTHER NATIVE I_K S

I_{K1} has not been previously described in the human myometrium. This current activated slowly and with a distinct delay and inactivated slowly. It had a negative apparent threshold of activation (between -60 and -50 mV), but with a shallow voltage-dependence of activation, such that the mid-point of activation was above 0 mV, and had a negative voltage-dependence of inactivation ($V_{0.5}$ between -61 mV and -67 mV). Unlike most cloned delayed rectifier-type K^+ channels (see Table 1.3.), I_{K1} was not blocked by 4-AP. It was, however, inhibited by TEA, with an EC_{50} close to 3 mM, and by clofilium with an EC_{50} close to 10 μ M.

Inactivation and TEA and 4-AP sensitivity

I_{K1} is similar in some of its properties to a component of the K^+ current recently described in pregnant and non-pregnant rat myometrium (Wang *et al.*, 1998). The C_1 component of

the whole cell I_K in that study was 59 % of the total current and had a $V_{0.5}$ of inactivation (using 10 s prepulses and test potential of +70 mV) of -60 mV. This current was also partially sensitive to TEA (2 mM) and 4-AP (> 5mM).

Native currents with similar characteristics to I_{K1} (4-AP-resistance and negative voltage-dependence of inactivation) have also been described in colonic smooth muscle of guinea-pig (Vogalis *et al.*, 1993) and dog (Thornbury *et al.*, 1992b; Carl, 1995), as well as in rat hippocampus (Segal & Barker, 1984). In their study, Vogalis *et al.*, (1993) also used TEA (5 mM) to block the Ca^{2+} -activated current, but used 2 s conditioning pre-pulses in availability experiments to derive a $V_{0.5}$ of -55 mV, compared to the more negative value in the present study using 10 second pre-pulses. Assuming the two currents are related, this discrepancy might be due to the very slow inactivation rate of this current such that a true steady state would not have been reached after 2 s, hence shifting the inactivation curve to the right. The 4-AP insensitive current described in the canine colon (Thornbury *et al.*, 1992b; Carl, 1995) also had a $V_{0.5}$ of close to -65 mV and was TEA-sensitive (EC_{50} 7.7 mM) but inactivated much more rapidly than I_{K1} . In the present study the availability of I_{K1} was not significantly influenced by partial block with either 1 or 10 mM TEA. The I_K described in rat hippocampus (Segal & Barker, 1984) had an activation threshold of -50 mV, a $V_{0.5}$ of inactivation of -63 mV, an EC_{50} for TEA of 10 mM and was insensitive to 4-AP.

Non-inactivating component of I_{K1}

The “non-inactivating component” of I_{K1} measured at the +60 mV test pulse during the 3-step inactivation protocol (30-43 % of total) also mirrored that of the currents described by Vogalis *et al.*, (1993) and Carl (1995), where the 4-AP-resistant currents inactivated by only 50 % and 80 %, respectively. Assuming that the currents described in these three studies do in fact share the same inactivation properties, then the differences in size of the non-inactivating component may result, at least in part, from the different conditioning

pulse durations used (2 s in Volgalis *et al.*, (1993), 10 s in the present study, and 20 s in Carl (1995)).

The absence of a substantial outward current resistant to 1 mM TEA in cells held for several minutes at 0 mV and the complete block of I_{K1} by 100 μ M clofilium at this potential supports the suggestion that the true non-inactivating component of I_{K1} at 0 mV was very small or non-existent. At +60 mV the test potential used in availability experiments, and in the 3-step inactivation protocol used to identify I_{K1} and I_{K2} components of the K^+ current, however, the non-inactivating component was only partially blocked by 100 μ M clofilium. This may mean that only a proportion of the current remaining at +60 mV after 10 s pre-pulses to 0 mV represents non-inactivated I_{K1} , and the remainder some other current. There are at least four possible candidates for this other current, including residual $I_{K(Ca)}$, I_{Cl} , $I_{K,ATP}$ and an other unidentified voltage-gated K^+ current.

There is more likely to be a substantial residual component of $I_{K(Ca)}$ at +60 mV than at 0 mV, because of the positive activation threshold of $I_{K(Ca)}$ under these experimental conditions (Fig 6.5B). However, since 1 μ M paxilline appears to be as potent as 10 mM TEA in blocking $I_{K(Ca)}$ when the V_H was 0 mV, it is less likely that the majority of the non-inactivating component is residual $I_{K(Ca)}$. The size of the non-inactivating component appeared to be smaller when 1 mM TEA was present than when 1 μ M paxilline was present and yet smaller when 10 mM TEA was present. This difference, if genuine, could be explained by the TEA sensitivity of I_{K1} .

A significant contribution of I_{Cl} , which has been described in human and rat myometrium at the single channel level (Coleman & Parkington, 1987; Wang *et al.*, 1998), to the non-inactivating component of I_{K1} at +60 mV is unlikely because niflumic acid did not inhibit

this current. The presence of 0.5 mM Cd^{2+} in most experiments also prevented the outward movement of K^+ through Ca^{2+} channels contributing to this current.

As described in chapter 5, there is also a K_{ATP} -like current in HMSMCs. This was present in one cell when ATP was excluded from the pipette but not in another where 5 mM ATP was present. It is possible however, that a small component of the outward current at +60 mV is contributed by a K_{ATP} current that is relatively insensitive to ATP, as has been shown to exist in other smooth muscles (Yamada *et al.*, 1997).

If the non-inactivating component is not entirely I_{K1} (or I_{K2}) and is actually represented partly by a distinct current, it is therefore most likely that it is another voltage-gated current. The most probable candidate for this is the minK/ K_v LQT1 channel which is expressed in rat uterus, activates and inactivates extremely slowly and has a similar sensitivity to clofilium as I_{K1} . However, it is also partially 4-AP sensitive and less TEA-sensitive than I_{K1} (Boyle *et al.*, 1987a; Attali *et al.*, 1992).

Block by clofilium

The block of I_{K1} (and I_{K2}) by the class III antiarrhythmic drug clofilium was concentration-dependent (EC_{50} between 10 and 30 μM). It was also time dependent, as illustrated by the greater block by 3 μM at the end of 10 second pulses to 0 mV than after 200-300 ms and the apparent enhancement of current decay during the 10 second pulses. The concentration-dependency resembles that described for the block of the cloned human delayed rectifier channels $\text{K}_\text{v}1.3$ and $\text{K}_\text{v}1.5$, with approximate EC_{50} s of 50 and 60 μM , respectively (Attali *et al.*, 1992; Malayev *et al.*, 1995). The enhanced decay of $\text{K}_\text{v}1.5$ current induced by clofilium was not dependent on membrane potential and the availability was not affected (Malayev *et al.*, 1995), thus suggesting that clofilium blocks delayed rectifier currents in a use-dependent, open channel-selective manner. In the present study, clofilium blocked the current remaining in the presence of 10 mM TEA and 5 mM 4-AP

equally at membrane potentials between -40 mV and 0 mV but significantly less at +60 mV. If the block of I_{K1} by clofilium is not voltage-dependent, then this confirms the above discussed assumption that another current may be activating at potentials above 0 mV. I_{K1} does not resemble either $K_v1.3$ or $K_v1.5$ in any of its other properties.

6.2.3. SIMILARITIES BETWEEN I_{K1} AND $K_v2.1$.

I_{K1} shares many of its electrophysiological properties with the $K_v2.1$ channel (see tables 1.2. and 1.3.) which is expressed in and has been cloned from rat and human tissues and characterised in transfection systems. $K_v2.1$ activation is voltage-dependent and activates slowly with a noticeable delay, which is more pronounced at negative membrane potentials (Pak *et al.*, 1991a; Shi *et al.*, 1994; Klemic *et al.*, 1998). This characteristic appears to be shared by all members of the *shab*-related K_v2 family of channels. The threshold of activation ranged between -30 mV and -50 mV (Pak *et al.*, 1991a; Shi *et al.*, 1994; Klemic *et al.*, 1998) and the $V_{0.5}$ of activation was 0 mV (Klemic *et al.*, 1998). The inactivation kinetics are also slow but with a $V_{0.5}$ of inactivation on average more positive than that of I_{K1} (between -30 and -45 mV; (Pak *et al.*, 1991a; Shi *et al.*, 1994; Klemic *et al.*, 1998). Klemic *et al.* (1998) recently showed that the voltage-dependence of inactivation of rat $K_v2.1$ was U-shaped with a maximum near to 0 mV, and that the speed of inactivation in long pulses was dependent on the amplitude of the depolarisation (90 % inactivation after 19 s at 0 mV but only 30 % inactivation after 19 s at +80 mV). They also showed that recovery from inactivation of this channel was > 10-fold faster than inactivation but that the channel inactivated faster during rapid repetitive pulses than during a single long pulse, concluding that it inactivates faster from a closed state than from the open state.

The sensitivity of cloned $K_v2.1$ to TEA and 4-AP is also similar to that of I_{K1} , although there was some variability between studies. The EC_{50} of $K_v2.1$ for TEA was between 3 and 10 mM and the EC_{50} for 4-AP was between 3 mM and >100 mM (Pak *et al.*, 1991a;

Kirsch & Drew, 1993; Shi *et al.*, 1994; Kukuljan *et al.*, 1995; Deal *et al.*, 1996; Patel *et al.*, 1997).

The characteristics of K_v2.1 are also influenced by co-expression with members of other, electrically silent, α -subunit families. For example, co-expression with K_v5.1 or K_v6.1 slowed channel deactivation, slowed inactivation at potentials between 0 and +40 mV but speeded it up at potentials between -30 and 0 mV, and speeded up the recovery from inactivation of K_v2.1 (Kramer *et al.*, 1998). Also, co-expression with K_v9.3 caused a leftward shift in the activation (from -30 to -50 mV) and inactivation ($V_{0.5}$ from -30 to -45 mV), slowed deactivation, and reduced the sensitivity of K_v2.1 to TEA (EC₅₀ from 3 to 9.5 mM) and 4-AP (EC₅₀ from 4.5 to 32 mM) (Patel *et al.*, 1997). The co-expression of K_v2.1 with K_v9.3 (both of which are expressed in rat pulmonary artery myocytes) also conferred ATP-sensitivity to the channel, wherein removal of intracellular ATP inhibited channel activity, suggesting a possible function for this channel in hypoxic vasoconstriction of the pulmonary circulation (Patel *et al.*, 1997).

6.2.4. CHARACTERISTICS OF I_{K2}

The second component of the delayed rectifier K⁺ current in HMSMCs, I_{K2} , was 4-AP-sensitive (approximate EC₅₀ of 1 mM) displaying an inactivation $V_{0.5}$ of -30 mV, a threshold of activation between -30 mV and -20 mV and a rate of inactivation much faster than I_{K1} . The availability of this current was similar to the sustained component of the 4-AP sensitive current described in human non-pregnant myometrium (Erulkar *et al.*, 1993) and to the C₂ component of the whole cell K⁺ current in pregnant and non-pregnant rat myometrium (Wang *et al.*, 1998), which was on average 23 % of the total K⁺ current and had a $V_{0.5}$ of inactivation of -21 mV. The C₂ current of Wang *et al.* (1998) had a similar sensitivity to TEA as C₁ (> 2 mM) and the I_{K1} current of the present study, but its 4-AP sensitivity was not clearly separated from that of C₁. The faster activation and inactivation of I_{K2} compared to I_{K1} and its sensitivity to 4-AP suggest that the channel or channels

responsible for I_{K2} in the myometrium are likely to be delayed rectifier-type members of the K_v1 channel family (see Tables 1.2 and 1.3), with the possible exception of $K_v1.2$ and $K_v1.5$, which are both very insensitive to TEA ($EC_{50} > 100$ mM) (Grissmer *et al.*, 1994; Overturf *et al.*, 1994).

6.2.5. DISTRIBUTION OF I_{K1} AND I_{K2}

The relative expression of the voltage-gated K^+ currents described in this study varied from one cell to the next, most cells possessing either I_{K1} or I_{K2} as the predominant voltage-gated current. Whether this means that different myocyte cell types or cells in a different state of differentiation express different K^+ currents is not known. Other influencing factors, such as age, are possible but unlikely, since cells with either I_{K1} or I_{K2} were often obtained from the same myometrial specimen. This finding clearly contrasts with that in the pregnant rat myometrium, where the two components of the K^+ current corresponding to I_{K1} and I_{K2} , C_1 and C_2 , both appeared to be expressed in all cells, as shown by the clearly biphasic voltage-dependence of inactivation of that current (Wang *et al.*, 1998).

6.2.6. POSSIBLE PHYSIOLOGICAL RELEVANCE OF I_{K1} AND I_{K2}

The excitability and contractile rhythmicity of the myometrium are dependent on the myometrial resting membrane potential and slow wave/action potential generation, respectively (Lodge & Sproat, 1981; Harding *et al.*, 1982; Parkington *et al.*, 1988; Buhimschi *et al.*, 1997). In smooth muscle, K^+ channels are believed to be important determinants of their resting membrane potential (Jmari *et al.*, 1986; Wilde & Marshall, 1988; Anwer *et al.*, 1993), modulating the amplitude and frequency of slow wave depolarisations (Wilde & Marshall, 1988) and modulating action potential spike frequency and duration (Jmari *et al.*, 1986; Amedee *et al.*, 1987; Wilde & Marshall, 1988). Even though it is difficult to extrapolate from data obtained under voltage-clamp in single

isolated myocytes to spontaneous electrical activity in strips of contracting muscle *in vitro* or the whole uterus *in vivo*, the electrophysiological and pharmacological characteristics of I_{K1} and I_{K2} described in the present study may suggest functions for these K^+ currents in myometrial electrical activity and contraction.

Resting potential and slow waves

The data presented in Fig 6.6 suggests that I_{K1} may have a window current between -60 and -20 mV with a maximum steady state current amplitude at about -36 mV. This means that channels responsible for this current are likely to be open, and therefore may contribute to the resting membrane potential (which in pregnant human myometrium is somewhere between -45 and -65 mV, Table 1.1.). Furthermore, this current will also be expected to flow during the upstroke of the slow wave, which is usually a depolarisation of 2-10 mV from the resting level (Nakao *et al.*, 1997; Parkington *et al.*, 1999), thus tending to drive the membrane potential back to the resting level, slowing the rate of depolarisation and affecting the frequency of action potential bursts. The activation threshold of I_{K2} (between -30 and -20 mV) in contrast, suggests that this current is less likely to contribute to the resting membrane potential or the upstroke of the slow wave.

Action potentials

The regenerative action potential depolarisations will serve to activate the channels responsible for I_{K1} and also I_{K2} . The deactivation kinetics of I_{K1} and I_{K2} were not determined in the present study, but if I_{K1} deactivation was found to be as slow as the cloned $K_v2.1$ channel (Kramer *et al.*, 1998), this, together with its slow inactivation kinetics would indicate a role in the spacing of the repetitive spikes within a burst, or the duration of a plateau-type action potential, and perhaps even ultimately terminating the slow wave depolarisation upon which the action potentials are superimposed. Even though I_{K2} inactivation is faster than that of I_{K1} , it is still likely to remain for the duration of a

typical action potential burst and would therefore also be expected to contribute to action potential repolarisation and slow wave termination.

Very few studies have evaluated the effect of blockers of voltage-gated K^+ channels on myometrial electrical activity or contraction. It has been shown that 4-AP depolarises strips of pregnant rat myometrium (Wilde & Marshall, 1988), and TEA (at 2 mM, a concentration that partially blocks I_{K1} and I_{K2}) depolarises strips of pregnant human myometrium (Parkington *et al.*, 1999). The effect of 4-AP however, may not only be an effect on I_{K2} , but also on I_{KA} , which may exist at or near the resting membrane potential (see chapter 5). The involvement of I_{K1} in myometrial electrical activity cannot be accurately determined until a specific blocker is found and the only known selective blocker of $K_v2.1$ is hanatoxin, a tarantula spider venom (Swartz & MacKinnon, 1995), which at the moment is not commercially available. In future studies, molecular biology techniques will be used to attempt identification of the channel or channels responsible for I_{K1} and, if it proves to be selectively expressed in the myometrium, or at least limited to rhythmic tissues, development of drugs which selectively modulate its activity, may lead to a new approach to the selective control of myometrial activity.

Chapter 7

Summary, Conclusions and Future Directions

7.1. CALCIUM CHANNEL CURRENTS

T-and L-components of I_{Ca}

Low threshold (T-type) and high threshold (L-type) voltage-gated calcium channel currents (I_{Ca}) are abundant in pregnant human myometrium, whereas voltage-gated sodium channel currents (I_{Na}) are very scarce. This contrasts with the rat, where I_{Na} is abundant and T-type I_{Ca} is scarce. The electrophysiological characteristics of the L-component current are well suited for a role of this current in action potential generation and these data are consistent with the effectiveness of L-type Ca^{2+} channel antagonists, but not Na^+ channel antagonists, at inhibiting spontaneous or agonist-induced myometrial contractions *in vitro* or *in vivo*. The electrophysiological characteristics of the T-type current suggest that it may also be involved in slow wave depolarisation or action potential generation. This latter hypothesis may be tested by the use of T-selective Ca^{2+} channel antagonists (such as mibefradil) in contraction and membrane potential experiments.

Oscillations and block by oxytocin

The spontaneous oscillations in current amplitude that occur in some cells, apparently irrespective of charge carrier (Ca^{2+} or Ba^{2+}) or the degree of intracellular Ca^{2+} buffering either indicate a resistance of the intracellular Ca^{2+} stores to depletion and the limitations of intracellular buffering methods used, or suggest that myometrial Ca^{2+} channels may be under a form of cyclical regulation by intracellular factors as well as, or other than, $[Ca^{2+}]_i$. Alternative mechanisms which may be investigated include protein kinase-dependent phosphorylation-dephosphorylation of the channel or its interaction with G protein $\beta\gamma$ subunits, as shown to occur in other cell types. Similarly, the inhibition of I_{Ca} or I_{Ba} by oxytocin occurs either indirectly via IP_3 -induced Ca^{2+} release from the SR or via other receptor-G protein transduction mechanisms.

Nimesulide

The COX-2 selective cyclooxygenase blocker nimesulide inhibits spontaneous myometrial contractions and Ca^{2+} channel currents *in vitro*, but at concentrations higher than those required to inhibit COX-2 activity in fetal membranes. The block of Ca^{2+} current in isolated myocytes by nimesulide occurs independently of endogenous prostaglandin production, indicating that the inhibition of myometrial contractions *in vitro* is predominantly via Ca^{2+} channel blockade. This blockade may also contribute to the tocolytic action of nimesulide *in vivo*. The enhanced potency of resting block and enhanced voltage-dependence of block by nimesulide at lower pH implies that its Ca^{2+} antagonistic properties may be enhanced in the acidotic myometrium during the contractions of labour, thus potentially improving its tocolytic efficacy and avoiding the serious cardiovascular side effects associated with conventional Ca^{2+} channel antagonists, as well as the serious fetal side effects associated with COX-1 selective, or non-selective COX inhibitors, such as indomethacin.

7.2 POTASSIUM CHANNEL CURRENTS

A-like Current

Many myocytes from both pregnant and non-pregnant myometrium possess a transient 4-AP sensitive outward current, or A-current ($I_{K,A}$). In some cells this current overlaps with the inward calcium current (I_{Ca}) at membrane potentials between -40 mV and +10 mV. In such cells, $I_{K,A}$ is often larger than, and always activates faster than I_{Ca} , producing a net transient outward current over the whole membrane potential range. The voltage-dependence of activation and inactivation of this current is strongly sensitive to external Cd^{2+} concentration, as shown previously for Cd^{2+} and other divalent cations, including Ca^{2+} , in other smooth muscles. The electrophysiological characteristics of this current suggest that its expression may promote myometrial quiescence. Further comparisons between the K^+ currents of pregnant and non-pregnant cells and current clamp membrane

potential experiments are needed to test this hypothesis and determine whether $I_{K,A}$ contributes to the resting membrane potential or influences the upstroke of the action potential spikes.

Glibenclamide-sensitive current

Preliminary data demonstrated the existence in human myometrium of a glibenclamide-sensitive K^+ current that is activated by levcromakalim, probably corresponding to the $I_{K,ATP}$ current described in other smooth muscles, and probably responsible for part of the myometrial relaxant effects of drugs such as levcromakalim and pinacidil. Further studies are planned in which the nucleotide dependency of $I_{K,ATP}$, its contribution to resting membrane potential, and the effects of agonists and second messenger systems will be evaluated.

Delayed Rectifier Currents

Two delayed rectifier K^+ currents are described in the human myometrium. I_{K1} is a 4-AP insensitive voltage-gated K^+ current with a low-threshold of activation and inactivation, which activates and inactivates slowly and which is sensitive to clofilium and high concentrations of TEA. I_{K2} is a 4-AP-sensitive voltage-gated K^+ current with a high threshold of activation and inactivation, which activates and inactivates faster than I_{K1} but still much slower than $I_{K,A}$, and is as sensitive to clofilium and high concentrations of TEA as I_{K1} . In myocytes from pregnant women, the distribution of I_{K1} and I_{K2} was such that most cells expressed either I_{K1} or I_{K2} as the predominant current. I_{K1} and I_{K2} correspond to the 4-AP insensitive and 4-AP sensitive components of the K^+ current described in other rhythmic tissues, including rat myometrium. The electrophysiological properties of I_{K1} resemble in many respects the $K_v2.1$ channel, especially when it is co-expressed with electrically silent regulatory α -subunits $K_v5.1$, $K_v6.1$ and $K_v9.3$, which have been cloned

from rat and human tissues. The properties of I_{K1} also suggest possible functions in the control of myometrial resting membrane potential and slow wave depolarisation, and hence rhythmicity of myometrial contraction. Future work aims to test these hypotheses using voltage and current clamp electrophysiological experiments in isolated single cells, as well as contraction experiments in strips of myometrium, following the appropriation of selective blockers or activators of these currents. Molecular biology techniques will be used to determine whether $K_v2.1$ and electrically silent regulatory α -subunits are expressed in human myometrium and whether their expression changes during pregnancy and labour. The effects of agonists and second messenger systems on I_{K1} and I_{K2} will also be examined.

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PUBLICATIONS ARISING FROM THIS STUDY

1. SAWDY R., KNOCK G.A., BENNETT P.R., POSTON L., AARONSON P.I. (1998): Effect of nimesulide and indomethacin on contractility and the Ca^{2+} channel current in myometrial smooth muscle from pregnant women. *Br J Pharmacol* 125: 1212-17.
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